

UTILITY PATENT  
APPLICATION TRANSMITTAL

(Only for new nonprovisional applications  
under 37 CFR 1.53(b))

Attorney Docket No.

000152

Total Pages

First Named Inventor or Application Identifier

Zenoviy TKACHUK

Express Mail Label No.

Check Box, if applicable [ ] Duplicate

APPLICATION ELEMENTS FOR: COMPOUND, COMPOSITION  
AND METHOD FOR THE TREATMENT OF INFLAMMATORY  
AND INFLAMMATORY-RELATED DISORDERS

ADDRESS TO: Assistant Commissioner for Patents  
BOX PATENT APPLICATIONS  
Washington, D.C. 20231

1. [XX] Fee Transmittal Form (Incorporated within this form)  
(Submit an original and a duplicate for fee processing)

2. [XX] Specification Total Pages [61]

[ ] Drawing(s) (35 USC 113) Total Sheets [ ]

[XX] Oath or Declaration Total Pages [2]

a. [XX] Newly executed (original)

b. [ ] Copy from prior application (37 CFR 1.63(d))  
(for continuation/divisional with Box 17 completed).

i. [ ] Deletion of Inventor(s)

Signed statement attached deleting inventor(s) named in prior application,  
see 37 CFR 1.63(d)(2) and 1.33(b).

[ ] Incorporation by reference (useable if box 4b is checked)

The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under  
Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby  
incorporated by reference therein.

6. [ ] Microfiche Computer Program (Appendix)

7. [ ] Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)

a. [ ] Computer Readable Copy

b. [ ] Paper Copy (identical to computer copy)

c. [ ] Statement Verifying identity of above copies

**ACCOMPANYING APPLICATION PARTS**

8. [XX] Assignment Papers (cover sheet and document(s))

9. [ ] 37 CFR 3.73(b) Statement (when there is an assignee) [XX] Power of Attorney

Jc678 U.S. PTO  
09/534509  
03/24/00

# UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications  
under 37 CFR 1.53(b))

Attorney Docket No. 000152

First Named Inventor or Application Identifier

Zenoviy TKACHUK

PAGE 2 OF 3

10. ☐ English translation Document (if applicable)

11. ☐ Information Disclosure Statement ☐ Copies of IDS Citations

12. ☐ Preliminary Amendment

13. ☒ Return Receipt Postcard (MPEP 503)

14. ☒ Small Entity Statement ☐ Statement filed in prior application  
Status still proper and desired.

15. ☐ Claim for Convention Priority ☐ Certified copy(ies) of Priority Document(s)

a. Priority of \_\_\_\_\_ application no. \_\_\_\_\_ filed on \_\_\_\_\_ is claimed under 35 USC 119.

The certified copies/copy have/has been filed in prior application Serial No. \_\_\_\_\_.

(For Continuing Applications, if applicable).

16. ☐ Other \_\_\_\_\_

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☐ Division ☐ Continuation-in-part (CIP) of prior application no. \_\_\_\_/\_\_\_\_

FEE TRANSMITTAL	Number Filed	Number Extra	Rate	Basic . . Fee
The filing fee is calculated below				\$690.00
Total Claims	22 - 20	2	x \$18.00	\$36.00
Independent Claims	6 - 3	3	x \$78.00	\$234.00
Multiple Dependent Claims			\$260.00	
Basic Filing Fee				\$690.00
Reduction by 1/2 for small entity				- \$480.00
Fee for recording enclosed Assignment			\$40.00	\$40.00
TOTAL				\$520.00

UTILITY PATENT  
APPLICATION TRANSMITTAL

(Only for new nonprovisional applications  
under 37 CFR 1.53(b))

Attorney Docket No.

000152

First Named Inventor or Application Identifier

Zenoviy TKACHUK

PAGE 3 OF 3

[XX] A check in the amount of \$520.00 is enclosed to cover the filing fee of \$480.00 and the assignment recordation fee of \$40.00.

[ ] Please charge our Deposit Account No. **01-2340** in the total amount of \_\_\_\_\_ to cover the filing fee and the \_\_\_\_\_ assignment recordation fee. A duplicate of this sheet is attached.

[XX] The Commissioner is hereby authorized to charge payment for any additional filing fees required under 37 CFR 1.16 or credit any overpayment to Deposit Account No. **01-2340**. A duplicate of this sheet is attached.

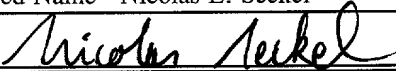
18. CORRESPONDENCE ADDRESS  
ARMSTRONG, WESTERMAN, HATTORI  
McLELAND & NAUGHTON  
1725 K Street, N.W. Suite 1000  
Washington, D.C. 20006  
Telephone: (202) 659-2930  
Facsimile: (202) 887-0357

SUBMITTED BY

Typed or Printed Name Nicolas E. Seckel

Reg. No. 44,373

Signature



Date: 3/24/00

NES/kah

# SMALL ENTITY DECLARATION

APPLICANT OR PATENTEE Zenoviy TKACHUK

SERIAL NO. \_\_\_\_\_ ☐ PATENT NO. \* \_\_\_\_\_ ATTORNEY DOCKET NO. 000152

1. ☐ FILED OR ISSUED \* \_\_\_\_\_

2. ☒ SUBMITTED HERewith

FOR COMPOUND, COMPOSITION AND METHOD FOR THE TREATMENT OF  
INFLAMMATORY AND INFLAMMATORY-RELATED DISORDERS

I (we) hereby declare that I (we) am (are) entitled to the benefit of small entity status with respect to the above-identified application or patent for purposes of paying reduced fees under 35 USC 41(a) & (b) to the U.S. Patent and Trademark Office.

☐ A. INDEPENDENT INVENTOR

I (we) qualify as (an) independent inventor(s) as defined in 37 CFR 1.9(c).

☐ B. INDIVIDUAL NON-INVENTOR

I would qualify as an independent inventor as defined in 37 CFR 1.9(c) if I had made the invention.

☒ C. SMALL BUSINESS CONCERN

I am ☐ THE OWNER ☒ AN OFFICIAL of the small business concern identified below and am empowered to act on behalf of the concern. The concern qualifies under 37 CFR 1.9(d) and 13 CFR 121.3-18. Rights under contract or law have been conveyed to and remain with the concern and are exclusive unless a checkmark is placed here ☐. All other rights belong to small entities as defined in 37 CFR 1.9.

☐ D. NON-PROFIT ORGANIZATION

I am an official empowered to act on behalf of the non-profit organization identified below. The organization qualifies under 37 CFR 1.9(e), sub-section \*. Rights under contract or law have been conveyed to and remain with the organization and are exclusive unless a checkmark is placed here ☐. All other rights belong to small entities as defined in 37 CFR 1.9.

I (we) acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I (we) declare under penalty or perjury under the laws of the United States of America that the foregoing is true and correct.

A. INDEPENDENT INVENTOR(S) B. INDIVIDUAL NON-INVENTOR(S)

\_\_\_\_\_  
Name

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

C. BUSINESS CONCERN D. NON-PROFIT ORGANIZATION

BioCell Laboratories, 1250 I Street NW Suite 1105, Washington, D.C. 20005

Name of Concern or Organization

Address

By Zenoviy TKACHUK

Name of Person Signing

  
Signature

\_\_\_\_\_  
Chairman of the Board

Title

03.23.2000  
Date

# **COMPOUND, COMPOSITION AND METHOD FOR THE TREATMENT OF INFLAMMATORY AND INFLAMMATORY-RELATED DISORDERS**

## **FIELD OF THE INVENTION**

The present invention concerns a compound and a pharmaceutical composition for the treatment of inflammation and diseases accompanied by inflammatory processes, in particular inflammatory processes which affect cellular membranes. The present invention also concerns therapeutic methods to ameliorate or prevent symptoms of inflammatory processes.

## **BACKGROUND OF THE INVENTION**

### **Inflammatory Processes**

Inflammation is generally accompanied by changes in the metabolism of arachidonic acid, metabolism of nitric oxide, and creation of free radicals. Anti-inflammatory non-steroid drugs (NSAIDS), such as aspirin, can block certain links of an inflammatory process, but these drugs cannot stabilize damaged cellular membranes, which makes their influence on an inflammatory process limited and insufficient.

Inflammation is a localized reaction of live tissue due to an injury, which may be caused by various endogenous and exogenous factors. The exogenous factors include physical, chemical, and biological factors. The endogenous factors include inflammatory mediators, antigens, and antibodies. Endogenous factors often develop under the influence of an exogenous damage. An inflammatory reaction is inevitably followed by an altered structure and penetrability of the cellular membrane. At the tissue and organ level, inflammation is indicated by pain, swelling, reddening, increased temperature, and a lost function in some cases. Inflammation begins with a sub-lethal damage and terminates either with a complete recovery or long-term tissue ruination. There is no recovery from an injury without an inflammation.

An immediate response to a tissue damage is realized via mediators, which are released due to the exocytosis or lysis of cells. The main inflammatory mediators are compounds of the kinine and fibrinolytic systems, the complement system, metabolites of arachidonic acid, vasoactive amines, and other chemical compounds. The chemical mediators of inflammation include: histamine, serotonin, prostaglandins, CGRP, nitric oxide, among others.

An important role in inflammations is played by various reactive oxygen-containing species. These compounds are synthesized when oxygen transforms them into very dangerous forms, producing free radicals, which are atoms and molecules with unpaired electrons. Different free radicals have different activity levels.

The launch of an inflammation is influenced by various exogenous and endogenous agents. Endogenous factors, namely, mediators, antigens, and autogens define the nature and type of the inflammatory reaction, especially its course in the zone of injury. In the case where a tissue damage is limited to the creation of mediators, an acute form of inflammation develops. If immunologic reactions are also involved in the process, through the interaction of antigens, antibodies, and autoantigens, a long-term inflammatory process will develop. Various exogenous agents, for example, infection, injury, radiation, also provide the course of inflammatory process on a molecular level by damaging cellular membranes which initiate biochemical reactions.

Inflammatory processes rely on the metabolism of arachidonic acid, which converts to prostaglandines (PG), tromboxanes (TX), and leukotrienes (LT). Prostaglandines, tromboxanes, and leukotrienes are the main participants of all inflammatory processes. There are two known ways of arachidonic acid cascade. The first way leads to the creation of prostaglandines  $G_2$  and  $H_2$ . This process is catalyzed by prostaglandin-cyclooxygenase. Cyclooxygenase catalyzes the production of  $PGA_2$ ,  $PGE_2$ ,  $PGD_2$ ,  $PGF_{2\alpha}$ , while tromboxane-synthesis with  $PGH_2$  produces tromboxane  $A_2$  ( $TXA_2$ ).

The cascade of metamorphoses of arachidonic acid, which is a product of membrane and phospholipase A<sub>2</sub>, is best known. Through its cyclogenase and lipoxygenase cascades, arachidonic acid turns into prostaglandins and leukotrienes, respectively. The cyclooxygenase way leads to the formation of two bio-active products: prostacycline (PGI<sub>2</sub>) and thromboxane (TXA<sub>2</sub>). These products are involved in many inflammatory effects: bronchoconstriction, vazodilation, vasoconstriction, platelet aggregation, analgesia, pyrexia, et al.

Another way of arachidonic acid metabolism with 5-lipoxygenase leads to the synthesis of leukotrienes: LTA<sub>4</sub>, LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, and LTF<sub>4</sub>. These leukotrienes have a powerful anti-inflammatory and bronchoconstrictor action, and they play an important role in vascular penetrability. Besides, leukotrienes are known as potential chemotactic factors; they increase the migration of WBC and have a great influence on the slow-releasing substance of anafilaxis (SRS-A).

Prostaglandines can play an important role in the development of systemic inflammatory reactions. In rheumatic arthritis, large quantities of PG and LT in the synovial liquid support the development of an inflammatory process and demineralization of bone tissue surrounding joints. Leukotrienes are known to be the main pathophysiological mediators of inflammatory reactions. They influence, to a greater degree than prostaglandines, the penetrability of vessels and the adhesion of leukocytes to vessel walls as well as the development of edema.

Prostaglandines effectively regulate the aggregation of platelets. PGE<sub>1</sub> is a powerful inhibitor of platelets aggregation, while PGE<sub>2</sub>, which is normally released from platelets, stimulates this process. However, the most important role in blood coagulability is played by PGI<sub>2</sub>, or prostacycline, which is synthesized in blood vessel walls by arachidonic acid. It is the most powerful inhibitor of platelets aggregation, which has vasodilator properties. Thromboxane, which is synthesized in platelets, has an opposite action.

When endothelium is damaged, the adhesion of platelets with subendothelium tissue and the aggregation of platelets is initiated. The main role in this process is played by thromboxane  $A_2$ . Prostaglandin  $I_2$ , on the contrary, inhibits the aggregation of platelets. Therefore, the proportion of  $PGI_2$  and  $TXA_2$  is crucial for the process of coagulation.

Further, a special role in the process of recovery from inflammation is played by nitrogen oxide (NO). This gas easily penetrates in different organs and tissues and, as a free radical, has a powerful reactivity. Nitrogen oxide is a potent vasodilator, neurotransmitter, and inflammatory mediator, which plays a significant role in asthmatic inflammation.

Nitrogen oxide is produced endogenously by L-arginine amino acid and NO-synthetase. There are three known forms of NO-synthetase, two of which are constituent, and one inducible. The inducible NO-synthetase, which is expressed in the epithelium cells, quickly increases its activity when anti-inflammatory cytokines (such as interleukin 1 beta (IL-1beta) and tumor necrosis factor (TNF-alfa) are released.

Nitrogen oxide has both positive and negative properties with respect to an inflammatory reaction. One important and potentially positive property is its ability to relax the smooth bronchial muscle, which results in bronchodilation. Its negative properties include the ability to help the inflammatory process by increasing chemotaxis neutrophils, monocytes, and eosinophils with the help of the guanine-monophosphate-dependent mechanism. It is believed that nitrogen oxide inhibits adhesion of leukocytes to vascular endothelium and bronchial epithelium.

NO plays an important biological role in defining basal vascular tonus, regulating contractions of myocardium, and modulating the interaction between thrombocytes and vascular walls (Zhou Q., Hellermann G.R., Solomonson L.P., Nitric oxide release from resting human platelets, *Thromb.Res.*, 1;77(1):87-86; 1995). The role of thrombocyte activation in the pathogenesis of various thrombo-vascular conditions in



humans and evidence about decreased NO-mediated effects in hypertension (Calver A., Collier J., Moncada S., Vallance P., Effect of local intra-arterial NG-monomethyl-L-arginin in patients with hypertension: the nitric oxide dilator mechanism appears abnormal, J.Hypertens., 10(9):1025-1031; 1990), diabetes (Calver A., Collier J., Vallance P., Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes, J.Clin.Invest., 90(6):2548-2554; 1992), and atherosclerosis (Drexler H., Zeiher A.M., Meinzer K., Just H., Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patient by L-arginine, Lancet., 21-28; 338(8782-8783); 1546-1550; 1991) suggests that drugs which increase the activity of NO-synthetase may effectively be used in treatment of patients. Human thrombocytes are capable of synthesizing nitric oxide. Large quantities of nitric oxide, for example, in the cells of endothelium, may be produced by intact thrombocytes, as well as by stimulated thrombocytes. Hence, nitric oxide of thrombocyte origin plays an important role in the support of vascular homeostasis and other NO-sensitive processes. (Zhou et al.,1995).

Beside some common features, inflammatory processes in each individual case have certain distinctions related to the peculiarities of functioning of the body organ and to the factors which caused the impairment: i.e., viruses, microorganisms, injuries, poisoning, etc.

For example, one of the common mechanisms of heart diseases, including acute infarct myocarditis, is a malfunction of the structure and function of the membrane of heart cells. As a result, the synthesis of leukotrienes, thromboxanes, etc., which have vasoconstrictor, arrhythmogenic, hemottractive and pro-aggregate action, increases (Bangham A.D., Hill M.W., Miller N., Preparation and use of liposomes as model of biological membranes, Methods in Membrane Biology, Acad. Press, V.1, N.Y., P.1-68, 1974).

Another important factor in the pathogenesis of heart impairments is the constrictor and hemoattractive (with regard to neutrophils) action of lipoxigenase derivatives  $LTC_4$ ,  $LTD_4$ ,  $LTB_4$  (Hoshida S, Kuzuya T., Nishida M., et al., *Amer.J.Cardiol*, 7; 63(10): 24E-2E; 1989; Lam B.K., Gagnon L., Austen K.F. et al., *J.Biol.Chem.*, 15; 265(23): 13438-1341; 1990; Svendsen J.N., Hansen P.R., Ali S. et al., *Cardiovasc.Res.*, 27(7): 1288-1294; 1993). Substances which can block this process can in turn reduce the size of necrosis at acute myocardial infarction and, therefore, significantly decrease the lethality in difficult cases of heart disease, such as gross myocardial infarction. At the same time, such substances can stabilize the membranes of heart cells. In addition, it is known that constrictor and hemoattractive effects during infarct are accompanied by an increased aggregation of platelets. Therefore, blocking this process also leads to a decrease of the size of impairment.

Further, disorders of the aggregate state of blood play an important role in the pathogenesis of various diseases. This is especially apparent in the pathogenesis of thrombo-vascular conditions in humans. It is known that a malfunction in the thrombo-vascular link of homeostasis is a key factor leading to disorders of the aggregate state of blood, by causing changes in the rheological properties of blood and triggering the formation of internal vascular aggregates. Thrombocyte-related injuries lead to failures in micro-circulation processes, which result in shortages of blood inflow to the tissue. At the initial stage of the formation of blood clots, platelets become activated and further undergo adhesion to the injured endothelium. Later on, they aggregate and an initial thrombocytic blood clot is formed.

Today, there is enough evidence of a close relation between inflammations, disorders in the aggregate state of blood, and cardio-vascular conditions (Anderson J.L. Carlqist J.L., et al., *Evaluation of C-reactive protein an inflammatory marker, and infectious serology as risk factors for coronary artery disease and myocardial infarction*, *J.Am. Coll. Card.*, 32: 35-41; 1998).

## Role of Cell Membranes in Inflammatory Processes

The functions of cell membranes and their relation to inflammatory processes has been documented. It is known that the plasmatic cellular membrane occupies a special place among the other membrane structures and performs such important functions as barrier and transportation, provides a contact with the outside environment for the cell, participates in the regulation of cellular homeostasis, supports signal mechanisms of this regulation, and defines the cell's individuality and wholeness. The structural organization, dynamics, and functions of erythrocytal membranes and various hemolysis patterns, such as osmotic, oxide, immune (induced by hemolytic viruses, toxins, complement), detergent hemolysis, photohemolysis, etc., are well studied (see, e.g., **Bashford C.L., Alder G.M., Menestrina G., et al., Membrane damage by hemolytic viruses, toxins, complement, and other cytotoxic agents. A common mechanism blocked by divalent cation. J. Biol. Chem., 15; 261(20): 9300-9308, 1986; Osorie e Castro V.R., Ashwood E.R., Wood S.G., Vernon L.P., Hemolysis of erythrocytes and fluorescence polarization changes elicited by peptide toxins, aliphatic alcohols, related glycols and benzylidene derivatives, Biochim. Biophys. Acta ., 16; 1029(2): 252-258; 1990).**

It was demonstrated that pH variation in the outside environment upsets the balance of forces influencing the membrane, which leads to structural changes and changes of the aggregation degree of membrane proteins. Two types of membrane structural changes are distinguished: those caused by pH variation in the range 7.0-6.0, and those for pH levels below 4.5 (**Zavodnik I.B., Pileckaya T.P., Acid lysis of human erythrocytes, Biophizika., V.42, N.5, P.1106-1112, 1997**). In the latter case, the membrane becomes destabilized and erythrocytal lysis follows. It is known that at pH 4.7, pores are formed in glycocalyx erythrocytal membranes (**Arvinte T., Cudd A., Schulz B., Nicolau C., Biochim. Biophys. Acta., 19; 981(1): 61; 1989**). In particular, decreased pH levels of the environment change the confirmation, package type, and mobility of

phospholipids in model membranes. Thus, aggregation of membrane proteins, denatured due to a decreased pH, is the reason for membrane damages and acid lysis in erythrocytes.

The pattern of erythrocytal hemolysis by HCl was proposed based on the cooperative protonation of some center located in stroma or on the membrane of erythrocyte with a subsequent creation of pores, sufficient to release hemoglobin. By studying the mechanism and pattern of the acid hemolysis process, information about the structural organization of the membrane and membrane-stabilizing actions can be obtained.

The best known endogenous stabilizers of hemolysis in erythrocytes (osmotic hemolysis is the best-studied) are albumin of blood plasma, metallic ions  $K^+$ ,  $Na^+$ ,  $Mg^{2+}$  and, especially,  $Ca^{2+}$ , which modulate the canals of plasmatic erythrocytal membranes, possibly including the proton canal (Anderson D.R., Davis J.L., Carraway K.L., **Calcium-promoted changes of the human erythrocyte membrane. Involvement of spectrin, transglutaminase, and a membrane-bound protease. J. Biol. Chem., 10; 252(19): 6617-6623, 1977**), cholesterol adsorbed on the surface of erythrocytes (Hui S.W., Stewart C.M., Carpenter M.P., Stewart T.P. **Effects of cholesterol on lipid organization in human erythrocyte membrane, J. Cell. Biol., 85(2): 283-291; 1980**), and polyamines, which bind with the fatty-acid residues of membrane phospholipids (Rennert O.M., Shukla J.B., **Polyamines in health and disease Advances in Polyamine research, Raven Press, V.2, N.Y, P.195-21, 1978**). The best known activators of endogenous hemolysis in erythrocytes are long-chain fatty acids (Rybszynska M., Csordas A., **Chain length-dependent interaction of free fatty acids with the erythrocyte membrane, Life Sci., 44(9): 625-632; 1989**), and especially free radicals of oxygen and nitrogen (Sato Y., Kamato S., Takahashi T. et al., **Mechanism of free radical-induced hemolysis of human erythrocytes: hemolysis by water-soluble radical initiator. 18; 34(28): 8940-8949; 1955; Sen T., Ghosh T.K., Chaudhuri A.K. Glucose oxidase-induced lysis of erythrocytes. J,Exp.Biol., 33;(1): 75-76; 1995;**

**Wollny T., Yacoviello L. Propagation of bleeding time by acute hemolysis in rats: a role for nitric oxide. Am. J. Physiol. 272(6): 2875-2884; 1997).**

In summary, there is evidence to suggest that the structure of the membrane is altered during inflammatory processes. However, the model of membrane damage in the inflammatory process has not been used for screening drugs and treating or preventing inflammation and inflammatory-related disorders.

#### Present Drugs Unsatisfactory

The present anti-inflammatory drugs are unsatisfactory because the difficult and various biochemical reactions involved in inflammations and the lack of reliable information about inflammatory pathogenesis complicate the experimental choice of pharmacological compounds capable to regulate inflammation. Thus, drugs are selected to have an effect on individual components of an inflammation. So far, there is no drug able to regulate most of the components of any inflammatory reaction.

Most of the known non-steroid anti-inflammatory drugs (NSAIDS) selectively influence certain phases of this pathological process. First, they influence the penetrability of blood vessels, which is often altered in acute inflammations, and various cell reactions, which are common for chronic inflammations. Also, many NSAIDS influence metabolism through the mechanism of free radicals.

The initial screening of anti-inflammation processes typically uses three groups of methods. First, the influence of drugs on easily-identifiable inflammatory symptoms is studied. These include swelling, hyperemia, necrosis, etc. A more advanced analysis includes experimental therapy methods, using model arthritis, carditis, etc., which are similar to human ailments. The third stage involves analysis of how the drug influences certain metabolic ways.

After the metabolism of arachidonic acid was studied in detail, many anti-inflammatory compounds, whose action was to regulate the formation of such metabolic products, were proposed. In most cases, such drugs act as inhibitors of the metabolic

enzymes of arachidonic acid. One example is the anti-inflammatory pharmacological combination of cyclooxygenase 2 inhibitor and leukotriene A<sub>4</sub> hydrolase inhibitor (Isakson, P.C., Anderson G.D., Gregory, S.A., **Treatment of inflammation and inflammation-related disorders with a combination of a cyclooxygenase-2 inhibitor and a leukotriene A<sub>4</sub> hydrolase inhibitor, United States Patent No. 5,990,148, Nov. 1999**). A similar approach was proposed on the basis of analogues of pyrimidines, a component of nucleic acids (Connor D.T., Kostlan C.R., Unangst P.C., **2-heterocyclic-5-hydroxy-1,3-pyrimidines useful as antiinflammatory agents, United States Patent No. 5,240,929, Aug. 1993**). Since these compounds are the inhibitors of key metabolic ferments of arachidonic acid, 5-lipoxygenase and cyclooxygenase, the authors suggested their use as anti-inflammatory drugs suitable for treatment of a wide range of diseases, from allergenic conditions and rheumatoid arthritis to atherosclerosis and myocardial infarction. Other researchers recommended prostacyclin analogues for treatment of thrombocyte aggregation and bronchoconstriction (Haslanger M.F., **Prostacyclin analogs and their use in inhibition of arachidonic acid-induced platelet aggregation and bronchoconstriction, United States Patent No. 4,192,891, Mar. 1980**).

However, since an inflammatory process initiates many different metabolic cascades, the use of inhibitors or metabolic analogues of arachidonic acid does not allow to balance all such reactions and, hence, cannot regulate the complex inflammatory process in a satisfactory manner.

Further, aspirin, which has been used in applied medicine for a long time, has also been proposed since it can block metabolic ferments of arachidonic acid. Inhibitors of prostaglandines, such as aspirin, quite effectively influence the inflammatory processes. For this reason, they are successfully used in clinics for the treatment of rheumatoid arthritis, osteoarthritis, and other similar inflammatory processes. Aspirin also has anti-coagulation properties, since it inhibits the synthesis of TXA<sub>2</sub>, and it influences at least partially the synthesis of PGI<sub>2</sub>. A daily dose of 3 g of aspirin is commonly used for

prevention of stenocardia, as a post-infarct and post-insult treatment, or for patients with a high risk of cardio-vascular conditions.

However, studies on the synthesis of TXA<sub>2</sub> and PGI<sub>2</sub> in vivo have shown that peroral administration of aspirin decreases the secretion of PGI<sub>2</sub> only for 2-3 hours, while the secretion of thromboxane is halted for 10 days (**Vesterqvist O., Measurements of the in vivo synthesis of thromboxane and prostacyclin in humans, Scand. J. Clin. Lab. Invest. 48(5): 401-407; 1988**). This author, as well as others (see, e.g., **Lorenz R.L., Boehlin B., Uedelhoven M.W., Weber P.C., Superior antiplatelet action of alternate day pulsed dosing versus split dose administration of aspirin, Am. J. Cardiol. 15; 64(18): 1185-1188; 1989**), not only show the difficulties in administering the right dose of aspirin, but also provide an experimental ground for the frequent side effects caused by aspirin during its long-term use.

Specifically, aspirin and other non-steroid anti-inflammatory drugs may be the cause of anaphylactoid reactions in sensitive individuals. The mechanism of these reactions is dose-dependent toxic-idiosyncratic, not immunologic. Also, aspirin is the most common cause of accidental poisoning. Children, treated by aspirin before poisoning, are also at great risk. Aspirin overdose, which occurs frequently, is difficult to correct. The effective aspirin dose for many diseases, including rheumatoid arthritis, constitutes 3-6.5 mg per day, which leads to irritations of the gastro-intestinal tract. Patients with gastro-intestinal conditions do not tolerate aspirin. Aspirin also causes erosion, bleeding stomach ulcers, diarrhea, and duodenum ulcers. Further, aspirin is commonly used in treatment for its anti-thrombocytic action, but it is badly tolerated and causes side-effects when taken for a long period of time. In addition, by inhibiting non-selectively cyclooxygenase, aspirin interferes with the synthesis of thromboxane, which is a powerful aggregant and vasoconstrictor, and may also lead to decreased levels of prostacycline, which is both anti-aggregant and vasodilator.

All these negative side-effects of aspirin and other NSAIDS motivate the search for new drugs which would have anti-inflammatory properties, but which are non-toxic in a wide range of concentration, have no side effects during a long-term use, and are capable of preventing and terminating inflammatory processes.

#### Pharmaceutical Use of Nucleic Acids

Nucleic acids are commonly used in pharmacology (Rothenberg M., Jonson G., Laughlin C. et al. **Oligodeoxynucleotides as anti-sense inhibitors of gene expression: therapeutic implications**, J. Natl. Cancer Inst., 18; 81(20): 1539-1544; 1989; Zon G., **Oligonucleotides analogues as potential chemotherapeutic agents**, Pharm. Res., 5; (9): 539-549; 1988). However, pharmaceutical uses for nucleic acids have not included inflammatory or inflammatory-related disorders. For example, Anderson et al., proposes the method of modulating the effects of cytomegalovirus infections with the help of an oligonucleotide, which binds with mRNA of cytomegalovirus, for treatment of cytomegalovirus infections in humans (Anderson K., Draper K., Baker B., **Oligonucleotides for modulating the effects of cytomegalovirus infections**, United States Patent No. 5,442,049, August 15, 1995). On the basis of a specific nucleic acid, which encodes the succession of 3' non-translated sector of protein kinase C, Boggs et al. propose a method for diagnosis and treatment of conditions, which are associated with protein kinase C alpha (Boggs R.T., Dean. N.M., **Nucleic acid sequences encoding protein kinase C and antisense inhibition of expression thereof**, United States Patent No. 5,681,747, Oct. 1997). Also, Yano et al. patented a DNA compound obtained from *Mycobacterium bovis* and *Bacillus subtilis* for treatment of stomach ulcers (Yano O., Kitano T., **Method for the treatment of digestive ulcers**, United States Patent No. 4,657,896, Apr. 1987).

In particular, it is known that ribonucleic acid (RNA), products of its partial hydrolysis, and synthetic poly-ribonucleotides have a wide range of bioactivity (Kordyum V.A., Kirilova V.S., Likhachova L.I., **Biological action of exogenous**



**nucleic acids, Visnyk ASC USSR, V.41, N.6, P.67-78, 1977).** They activate protein synthesis in cells (Sved S.C., **The metabolism of exogenous ribonucleic acids injected into mice, Canad.J.Biochem., V.43, N.7, P.949, 1965**) and have anti-tumor activity (Niu M.C., **Effect of ribonucleic acid on mouse acids cells, Sciens., N.131, P.1321, 1960**). RNA can increase antibody generation and decrease the inductive phase of antibody genesis (Johnson A.G., Schmidtke I., Merrit K. et al., **Enhancement of antibody formation by nucleic acids and their derivatives, in Nucleic acid in immunology, Berlin, P.379, 1968; Merrit K., Johnson A.G., Studies on the adjuvant of bacterial endotoxins on antibody formation, 6. Enhancement of antibody formation by nucleic acids, J.Immunol., V.94, N.3, P.416, 1965; Brown W., Nakono M., Influence of oligodeoxyribonucleotides on early events in antibody formation, Proc. Soc. Exper. Biol. Med., 5, V.119, N.3, P.701, 1967**). It was shown that certain increased or decreased immunologic indicators normalize under the influence of RNA. In the first place, this applies to T-lymphocytes, cooperation of T- and B-lymphocytes, activation of macrophage function, etc.

Further, exogenous RNA is used for the DNA synthesis in dividing cells and for the RNA synthesis in metabolizing cells. It was also determined that 2 hours after the introduction, exogenous RNA was included in the RNA of lymphocytes and macrophages (Enesco N.E., **Fate of 14C-RNA infected into mice, Exper. Cell Res., V.42, N.3, P.640, 1966**). Evidence suggests that yeast tRNA can be included into cells in the form of intact molecules (Herrera F., Adamson R.H., Gallo R.C., **Uptake of transfer ribonucleic acid by normal and leucemic cells, Proc.Nat.Acad.Sci.USA, 67(4): 1943-1950; 1970**).

It was determined by analytical methods that RNA is present in practically all membranes of animal cells (membranes of endoplasmic reticulum, mitochondrial, nucleic, and plasmatic membranes). Its content, depending on the type of tissue and on the method of membrane isolation, varies between 0,5 and 4% of the dry weight of the membrane.

Experimental results show that special membrane RNA exists in isolated membranes (Shapot V.S., Davidova S.Y., Liponucleoprotein as an integral part of animal cell membrans. Prog. Nucleic Acid Res. 11: 81-101; 1971; Rodionova N.P., Shapot V.S. Ribonucleic acid of the endoplasmatic reticulum of animal cells. Biochim et Biophys Acta, 24; 129(1); 206-209; 1966). The functions of membrane RNA are not fully understood.

The functions of membrane RNA in ribosome have been better studied. (Cundliffe E., Intracellular distribution of ribosoms and poliribosomes in Bacillus megaterriium. J.Mol.Biol., 28; 52(3): 467-481; 1970) Ribosomal RNA is contained in bacterial membranes, in the outer membranes of nuclei, inner and outer membranes of mitochondria, inner membrane of the Goldgi apparatus, which adjoins the plasmatic membrane, in the rugged endoplasmic reticulum, in different tissues in animals, humans, plants, microorganisms, and protozoa. It is possible that membrane glycolipids and glycoproteins, which contain N-acetylneuraminic acid, are involved in the formation of binding sites of ribosomal RNA in ribosomes, since membranes which are treated by neuronidase lose the ability to bind ribosomes. (Scott-Burden T., Hawtrey A.O., Preparation of ribosome free membranes from rat liver microsomes by means of lithium chloride. Biochem. J. 115(5): 1063-1069; 1969. Further, it is possible that binding sites of ribosomes and membranes are activated by the sexual hormones, and cancerogens damage this physiological mechanism. This conclusion is supported by decreased levels of membrane-bound RNA in the process of aging (Mainwaring W.J. The effect of age on protein synthesis in mouse liver. Biochem J. 113(5): 869-878; 1969) and after castration of animals (Tata J.R., The formation, distribution and function of ribosomes and microsomal membranes during induced amphibian metamorphosis. Biochem J. 105(2): 783-801, 1967). Extraction of spermine from a membrane leads to a separation of bound RNA from the membrane (Khawaja J.A. Interaction of ribosomes and ribosomal subparticles with endoplasmic reticulum

**membranes in vitro: effect of spermine and magnesium. Biochim. Biophys. Acta., 29; 254(1): 117-128; 1971).** When membranes are treated with RNA of native small ribosomes of myeloma cells, they separate from the membranes, while large native subunits of ribosomes remain bound with the membranes (Mechler B., Vassalli P., **Membrane-bound ribosomes of myeloma cells. I. Preparation of free and membrane-bound ribosomal fractions. Assessment of the methods and properties of ribosomes. J. Cell. Biol. 67(1): 1-15; 1975** . Also, the nucleotide components of various membrane enzymes, for example, polyA-RNA enzyme of phosphofructokinase, constitute a possible pool of membrane RNA (Hofer H.W., Pette D. **The complex nature of phosphofructokinase – a nucleic acid containing enzyme, Life Sci. 4(16): 1591-1596; 1965).**

However, nucleic acids, and in particular RNA, and compositions containing the same, have not been used to treat or prevent inflammatory or inflammatory-related disorders. In particular, most of the studies above rely on experiments *in vitro*. Further, none of these methods is directed to treating or preventing an inflammation or inflammatory-related disorder.

#### Need for New Drug

In view of the above, there is a need for new anti-inflammatory drugs which would regulate disorders of the aggregate state of blood and would have less negative effects than aspirin and other NSAIDS. In particular, since an inflammatory process in the initial stage is followed by alterations in the structure and functions of the membrane in the many cells involved in the inflammatory process, drugs are needed which, not only regulate all the components of an inflammatory metabolic cascade, but also stabilize membrane structures and functions in the involved cells. In particular, since the traditional therapy has little effectiveness in extensive infarcts, which are complicated by the cardiogen shock, there is a need for new drugs capable of stopping the destruction of cardiomyocytes.

## SUMMARY OF THE INVENTION

The present invention offers a compound, a pharmaceutical composition and a method for the treatment or prevention of inflammation and diseases accompanied by inflammatory processes. The compound is an active ingredient consisting of RNA, in particular RNA extracted from yeast. Yeast RNA is a heterogenous compound of low-polymeric RNA, which comprises various quantities of nucleotides, nucleotide polymers, and usually 5 to 25 nucleotides. Oligonucleotides and transport RNA with a great number of minor bases prevail in yeast RNA.

Since one of the common features of all inflammatory processes at a molecular level is altered penetrability and structure of membrane, the present invention was made using a method of selecting drugs based on their ability to stabilize cellular membrane in inflammations. Thus, by analyzing destructive mechanisms induced by various factors in plasmatic membranes and learning about the structural elements of their interaction, which provide the optimal organization of a cell, it is possible to select drugs having membrane-stabilizing action for applied medicine. Specifically, it has now been established that, since membranes contain low-molecular RNA which probably plays a membrane-stabilizing role, introduction into the body of exogenous low-molecular RNA leads to stabilization of disturbed membranes, such as, for example, membranes of cells involved in inflammatory processes.

Stabilization of the cell membrane by the compound of the present invention leads to the normalization of arachidonic acid metabolism and nitric oxide metabolism, which have a powerful anti-inflammatory action and are the main participants of all inflammatory processes, for example, rheumatoid arthritis, osteoarthritis, allergies (such as asthma), and other inflammatory conditions, such as pain, swelling, fever, psoriasis, inflammatory bowel disease, gastrointestinal ulcers, cardiovascular conditions, including ischemic heart disease and atherosclerosis, partial brain damage caused by stroke, skin

conditions (eczema, sunburn, acne), leukotriene-mediated inflammatory diseases of lungs, kidneys, gastrointestinal tract, skin, prostatitis, and paradontosis.

The yeast RNA is effective in decreasing the activity of iNOS in the course of an auto-immune process, both during its initiation and in the chronic stage. This property allows the usage of yeast RNA in pathological conditions which are accompanied by iNOS induction: diabetes, tumor, hepatitis, infections, neuro-degenerate diseases (Parkinson's disease, Alzheimer's disease, multiple sclerosis, encephalitis), and others.

In addition, the use of natural molecules of nucleic acids, such as the compound of the present invention, in large concentrations as pharmacological compounds causes no or little side effects, especially taking into account the fact that this compound constantly enters human and animal bodies with food.

Further, the present invention offers a method for the treatment of inflammation or inflammatory-related disorder comprising administering to a mammal in need of such treatment an amount effective to ameliorate the symptoms of inflammation or inflammatory-related disorder of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

Still further, the present invention offers a method of stabilizing damaged cellular membranes which comprises administering to a mammal having damaged cellular membranes an amount effective to stabilize said damaged cellular membranes of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

Still further, the present invention offers a method of normalization of NO-synthetase ability in a mammal which comprises administering to a mammal in need of such treatment an amount effective to normalize NO-synthetase ability in the mammal of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

Still further, the present invention offers a method of inhibiting oxidation of components of cell membranes of a mammal, which comprises administering to a mammal in need of such treatment an amount effective to inhibit oxidation of components

of cell membranes of the mammal of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

Still further, the present invention offers a method of inhibiting thrombocyte aggregation, which comprises administering to a mammal in need of such treatment an amount effective to inhibit thrombocyte aggregation of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

Also, the present invention offers a compound consisting of ribonucleic acid extracted from yeast, for example a *Saccharomyces cerevisiae* or a *Candida utilis*. Preferably, the ribonucleic acid has a nitrogen content of more than 14.5% by weight and a phosphorus content of more than 8,5% by weight, more preferably a nitrogen content of more than 14.7% by weight and a phosphorus content of more than 8,6% by weight, even more preferably a nitrogen content of more than 15.0% by weight and a phosphorus content of more than 9.0% by weight.

Further, the present invention offers a pharmaceutical composition for the treatment or the prevention of inflammation or inflammatory-related disorder, comprising ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

#### **DETAILED DESCRIPTION OF THE INVENTION**

A complex analysis of known nucleic acids was carried out using various *in vitro* and *in vivo* models. The models were chosen to correspond to certain types of inflammatory processes, both of common and immunologic origin. In the tests, the effects of ribonucleic acid (RNA), in particular yeast RNA, was compared to the effects of existing anti-inflammatory drugs over a wide range of anti-inflammatory activities.

## Summary of Experimental Models and Results

### 1. Model of Thrombocyte Aggregation *in Vitro*

An initial screening of exogenous nucleic acids was conducted *in vitro* on the model of aggregation of human thrombocytes induced by arachidonic acid (Born L.V.R. **The aggregation of blood platelets by difosphate and its reversal, Nature, V.94, P.327, 1962**). Exogenous DNA and RNA from prokaryotes and eukaryotes were analyzed. We used aspirin as representative of a standard anti-inflammatory drug.

It was demonstrated that aspirin inhibited the aggregation of thrombocytes induced by arachidonic acid to a certain level. Desoxyribonucleic acid obtained from chicken erythrocytes (DNA-CE) produced by "Reanal" (Hungary), inhibited thrombocytic aggregation within the range of aspirin. Further, DNA from cattle thymus (DNA-CT) produced by "Reanal" (Hungary), and transport RNA of *E.coli* (tRNA) produced by "Serva" (USA) inhibited aggregation of the induced thrombocytes almost twice. The highest inhibiting effect was demonstrated by total yeast RNA, which dramatically inhibited thrombocytic aggregation in a wide range of concentrations. Inhibition of thrombocytic aggregation by yeast RNA depended on the form (acid or its sodium salt), purity, and presence of protein. RNA-F with protein admixtures was less effective by a third. The sodium salt of yeast RNA-PN in high concentration was only half as effective, and did not act in low concentration.

Since the model of aggregation of thrombocytes induced by arachidonic acid is recognized for the selection of anti-inflammatory drugs, the results of this comparative test showed that nucleic acids, and especially RNA, in particular, yeast RNA, have pronounced anti-inflammatory properties.

### 2. Model of Acid Resistance of Erythrocyte Membranes *in Vitro*

Based on the recognition that destabilization of cellular membranes is the main indication of an inflammatory process, we used the model of acid resistance of erythrocyte membranes *in vitro* for the screening of membrane-protecting, and thus, anti-inflammatory

properties of the drugs. We chose rat erythrocytes to study the immune-stabilizing action of exogenous nucleic acids. We analyzed the reactions of erythrocytic membranes to the destructive influence of nitric oxide. We estimated the membrane-stabilizing action of exogenous nucleic acids and damaging actions of endogenous and exogenous nitrite anion by calculating the acid resistance of erythrocytes according to the kinetic method **(Terskov I.A., Hittelzon I.I., Chemical (acid) erythrogram method, Biophizika, 2(2): 259-266; 1957)**. The main idea of the method is to determine historical changes in the number of cells, which eventually become hemolyzed under the influence of weak acids. The lysis of erythrocytes in acid environment undergoes three stages: penetration of hydrogen ions (protons,  $H^+$ ) through the plasmatic membrane of erythrocytes, protonation of hemoglobin and membrane proteins, and, as a result, osmotic destruction of erythrocytes.

Using this method, we estimated the influence of exogenous nucleic acids on the kinetics of the penetration of protons through the erythrocytic plasmatic membrane, which depends on the membrane's nature. The speed of proton penetration in the cellular cytosol depends to a great extent on the oxidation status of the lipid component **(Kellogg E.W., Fridovich I., Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide J. Biol. Chem. 10; 252(19): 6721-6728; 1977)** and protein component, especially, the band 3 oxidation of plasmatic membranes and is defined by the activity  $[H^+]$ -ATP-ase, and the activity of various exchangers **(Sato Y., Kamo S., Takahashi T., Suzuki Y., Mechanism of free radical- induced hemolysis of human erythrocytes: hemolysis by water-soluble radical initiator, Biochemistry, 18; 34(28): 8940-8949; 1995; Lukacs G.L., Kapus A., Nanda A. et al, Proton conductance of the plasma membrane: properties, regulation, and functional role, Am. J. Physiol, 265(1 Pt 1): C3-C14; 1993)**.



Acid erythrograms were recorded by the kinetic method. In the *in vitro* tests, acid erythrograms were recorded in the presence of sodium nitrite (the damaging agent) and different concentrations of exogenous nucleic acids.

The *in vitro* tests, which used the oxide damage model of erythrocytes by nitrite anion, a stable metabolite of nitric oxide, demonstrated stabilizing and membrane-protector action of exogenous nucleic acids.

On the model of acid resistance of erythrocytic membranes, we tested the same set of preparations as in the model of thrombocytic aggregation.

Yeast RNA preparations demonstrated membrane-protecting properties in a wide range of concentrations. A more detailed analysis showed that the membrane-protector action of yeast RNA depends on their form (acid or sodium salt), purity, and the presence of protein. Well-purified ribonucleic acid RNA-P, whose erythmograms in the concentrations 10 and 100  $\mu\text{kg}$  corresponded to the norm, showed the highest effectiveness. Sodium salt of yeast RNA-PN was less effective, especially in the concentration 10  $\mu\text{kg}$ . Protein admixtures in RNA-F resulted in a complete loss of the membrane-stabilizing action. Other preparations, tRNA, DNA-CT, and DNA-EC destabilized erythrocyte membranes at the tested concentrations, which means that they cannot be used as anti-inflammatory drugs as advantageously despite their anti-inflammatory properties demonstrated on other models.

### 3. Model of Erythrocytal Auto-Immune Reaction in Rats

We used the model of acid injury of erythrocytal plasmatic membranes to study the membrane-stabilizing action of exogenous nucleic acids. Acid damages to the protein and lipid components of erythrocytal plasmatic membranes were tested *in vivo* in the process of development of an auto-immune reaction (adjuvant arthritis). The biosynthesis of nitric oxide, which is an active oxidizing agent, became activated and, especially, hemoglobin of erythrocytes (Eich R.F., Li T., Lemon D.D. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry*, 4; 35(22): 6976-6983; 1966; Huot A.E.,

Kruszyna H., Kruszyna R. et al., Formation of nitric oxide hemoglobin in erythrocytes co-cultured with alveolar macrophages taken from bleomycin-treated rats. *Biochem.-Biophys. Res. Commun.*, 15; 182(1): 151-158; 1992; Kosaka H., Harada N., Watanabe M. et al. Synergistic stimulation of nitric oxide hemoglobin production in rats by recombinant interleukin 1 and tumor necrosis factor. *Biochem. Biophys. Res. Commun.* 30; 189(1): 392-398; 1992). Nitric oxide, as well as hydrogen peroxide, plays a crucial role in the damage to cells, including blood cells, in the process of development of autoimmune reactions. The anti-inflammatory cytokines (gamma-interferon, IL-1) induce expression of the inducible isoform of NO-synthetase (iNOS).

We studied changes in the activity of NOS in rat blood in the development of autoimmune reaction (adjuvant arthritis) in order to evaluate the preparation's immune-modulating effect and to obtain information about possible levels of one of the most active oxidizing hemolytics, nitric oxide (in the form of its stable metabolite, nitrite anion). We calculated the activity of the enzyme NO-synthetase (NOS), which generates endogenous nitrite anion. These values characterize the protective effect of exogenous nucleic acids against the damaging influence of nitrite anion on erythrocytic membranes. Our focus on the changes in stability of erythrocytes in the process of autoimmune reactions is due to the large existing body of evidence supporting the immune-modulating properties of erythrocytes (Karalnik B.V., Erythrocytes, their receptors, and immunity, *Uspekhi Sovremennoy Biologii.*, V.112, N.1, P.52-61, 1992; Prokopenko L.H., Siplivaya L.E., Erythrocytes as modulators of immunologic reactions, *Uspekhi Phiziologicheskikh Nauk.*, V.23, N.4, P.89-106, 1992), which has resulted in the use of the term "erythrocytal immune system".

Development of the autoimmune process was accompanied by a substantial decrease of acid resistance of erythrocytes during the early stage and, on the contrary, by a

considerable excess over the norm during the final stage, in comparison with the resistance of normal erythrocytes.

Yeast RNA increased membrane stability, i.e., normalized the process of transportation of protons (which is attributed to the state of the protein and lipid components of erythrocyte plasmatic membranes) during the initial stage and kept it stable, close to the norm, during the following stages of autoimmune reaction.

Further, it was demonstrated that, during the development of an autoimmune process, activities of NOS in rat blood changed. During the initial and final stages, an increased activation of NOS in rat blood was evidenced. Yeast RNA decreased NOS activity, so that at the final stage, the activity was practically normal.

Also, development of the autoimmune process was accompanied by a substantial decrease of acid resistance of erythrocytes during the early stage and, on the contrary, by a considerable excess over the norm during the final stage, in comparison with the resistance of normal erythrocytes. Yeast RNA increased membrane stability during the initial stage, by normalizing the process of proton transportation, which is dependent on the state of the protein and lipid components of erythrocyte plasmatic membranes, and kept it stable, close to the norm, during the following stages of autoimmune reaction.

In view of the above, the protecting activities of yeast RNA as shown on the model of autoimmune process establish its ability to cure, not only allergic diseases, but other chronic inflammatory processes as well, such as arthritis, atherosclerosis, and other diseases that involve autoimmune reactions.

#### 4. Model of Swelling Induced by Carrageenan in Rats

To screen nucleic acid's anti-inflammatory action, we used a common model of inflammatory swelling of leg in mice provoked by a sub-plantar injection of carrageenan. Carrageenan-induced swelling is sensitive to the action of compounds which reduce capillary penetrability.

During the initial stage, a significant role in the mechanism of anti-inflammatory effect of carrageenan is played by kinine, while at the later stage, proteolytic ferments and

prostaglandins become more important. The carrageenan model has a slower development and is preserved for a sufficient time, which makes it possible to study the biochemical mechanism of the anti-inflammatory action of a drug. Therefore, we used this model to study the influence of yeast RNA on the synthesis of thromboxane and leukotriene. At the same time, we analyzed the influence of yeast RNA on NO-synthetase activity.

Analysis of the anti-inflammatory action of nucleic acids in the carrageenan model showed that they all have certain anti-inflammatory action. However, only yeast RNA in the concentration 10 mg of drug per mouse resulted in a 50% reduction of swelling. The concentrations of yeast RNA tested in mice represented 1 to 15 mg per mouse. Concentrations below 1 mg of yeast RNA preparation per mouse did not show any action. In concentrations above 15 mg, reduction of swelling was about 53-55%. Further, biochemical tests revealed a stabilizing influence of yeast RNA on the activity of NO-synthetase as well as on the quantities of thromboxane and leukotriene, which varied in the course of swelling process.

By contrast, aspirin, which was tested at the recommended therapeutic dose of 20 mg/kg, influenced swelling to a considerably smaller extend and did not show stabilizing properties at the level of biochemical metabolism.

#### 5. Model of Acute Ischemia in Rats

Further analysis of yeast RNA was conducted on the model of acute ischemia-reperfusion of myocardium in rats. This model is based on a common fundamental mechanism in the development of a variety of different heart conditions, which includes alteration of structures and functions of the membranes in endotheliocytes, cardiocytes, and other heart cells. This alteration results in the degradation of membrane phospholipids and the creation of highly effective bio-active compounds, such as leukotrienes or thromboxanes, which have coronaroconstrictor, arythmogen, chemoactive, and pro-aggregant action (Bangham A.D., Hill M.W., Miller N., Preparation and use of

**liposom as model of biological membranes, Method in Membrane Biology, Acad.Press, V.1, N.Y, P.1-16, 1974).**

As the tests demonstrated, yeast RNA, injected in rats intravenously in the concentration of 40 mg per rat, normalized heart function in acute infarcts. This was shown in a pronounced anti-arythmic action of the compound and a substantial decrease of the necrosis area in ischemized myocardium of heart. The drug almost completely normalized NO-synthetase activity in blood and in the border zone of ischemized heart. Yeast RNA injection normalized to a certain level the content of arachidonic acid in blood and heart of animals in acute infarctions. The injection of yeast RNA almost completely normalized the levels of eukosanoids in rat blood in ischemia cases. The activity of mieloperoxidase, the marker enzyme of neutrophils which helps to evaluate the preparation's anti-oxidant action, decreased almost twice in animals with infarct treated by yeast RNA.

The analysis of yeast RNA activity in the ischemia-reperfusion model in rats determined that the drug has a substantial stabilizing action in different cascades of inflammatory processes in the ischemized heart, which is expressed in its long-term anti-infarct action and a decreased size of the infarct area in myocardium.

On the basis of the study of yeast RNA action in ischemia-reperfusion of animal heart, we can conclude that yeast RNA has an anti-infarct action, or anti-inflammatory action in infarcts, through stabilization of the structure and function of membranes in endotheliocytes, cardiocytes, and other heart cells.

## **Experimental Procedures and Test Results**

### **Example 1. Method for Obtaining Yeast RNA**

#### **Example 1.1. Production of Yeast RNA**

From *Saccharomyces cerevisiae* was obtained RNA-D and from *Candida utilis* were obtained RNA-P, RNA-PN, and RNA-F. Yeast RNA extraction was conducted with a 10-12% solution of sodium chloride at 100-110°C. The RNA solution was separated from yeast sediment, cooled to 0°C and acidified to pH 1-2 by hydrochloric acid. Deposited RNA was rinsed by ethyl alcohol, dried and dissolved in water. The solution was brought to pH 8.0-8.2 by sodium hydroxide. The solution with added pancreatin was kept at 37-40°C for approximately 1 hour. The ferment was inactivated by boiling; afterwards, the solution was filtrated. RNA was sedimented by cooled ethyl alcohol, acidified by hydrochloric acid to pH 1-2, and dried. In this way, RNA-F was obtained. Further, the sediment was filtrated, rinsed in ethyl alcohol, and dissolved in water by adding sodium hydroxide to pH 6.2-6.5. RNA-PN was sedimented by alcohol. The sediment was filtrated and dried. RNA-P was educed from RNA-F by additional purification from protein by another pancreatin treatment and incubation for 1 hour at 37-40°C. Then, the ferment was inactivated by boiling for 5-10 min. The solution containing RNA-P was filtrated and sedimented by alcohol acidified to pH 1-2. The RNA-P sediment was filtrated, rinsed in ethyl alcohol and dried. The resulting compound has a grey-yellowish color.

Table 1.

## Chemical Analysis of Yeast RNA Preparations

Type	RNA-P	RNA-D	RNA-F	RNA-PN
Nitrogen content %	15.49	15.16	14.16	14.65
Phosphorus content %	9.05	8.6	8.2	8.54
Biuret reaction	(-)	(-)	(+)	(-)
DNA content %	1	1.1	1.2	1.1

The tested RNA (RNA-P and RNA-D) had the following properties as shown in Table 1: N  $\geq$  14.7%, P(total)  $\geq$  8.6%, protein (biuret reaction) – negative, DNA (colometric) - 2.0%, sugars (chromatography) – negative, polysaccharides (biological test) – negative.

Example 1.2. Absence of Toxicity

We established that yeast RNA-P and RNA-D are non-toxic. Single or multiple doses of yeast RNA in bio-active amounts (250 to 500 mg per 1 kg of body weight), taken intra-abdominally, did not lead to substantial changes in the quantity of peripheral lymphocytes in mice. Such changes would be a characterizing indicator for endotoxines.

Analogous results were obtained for intravenous introduction of nucleic acids. We tested variations in the quantity of peripheral leukocytes in rabbits 1-3 hours after 100 mg yeast RNA-P or RNA-D solution was injected intravenously. Intravenously injected solution of 0.85% NaCl was used as the standard of non-toxicity. It was demonstrated that, analogously to the standard, an injection of yeast RNA-P or RNA-D does not cause a variation in the number of leukocytes within 3 hours of the introduction. In animals, which took 0,85% solution of NaCl, the quantity of leukocytes was equal to  $13000 \pm 980$ , while those, who had RNA-P or RNA-D, showed accordingly  $12700 \pm 850$  and  $12900 \pm 980$ , which is not abnormal. When the rabbits received injections of 10 mg of proteus polysaccharide, the quantity of leukocytes decreased in 1 hour from  $13050 \pm 1100$  to  $2900 \pm 210$ , and remained at that level while the test lasted (3 hours). These results prove

the non-toxicity of yeast RNA. Further, when 100 mg of yeast RNA-P or RNA-D per 1 kg of body weight was given to rabbits intravenously, no acute-phase C-reactive protein was determined, which indicates that there was no endotoxic action.

In addition, yeast RNA is not pyrogenic, which was shown on rabbits. Temperatures were taken 4 times a day, with 2-hour intervals, in a group of rabbits for 2 days. On the third day, the rabbits were injected with 0.85% of NaCl, and the temperatures were taken again 1, 2, and 3 hours after the injection. On the sixth day, the rats were divided into 3 groups, two of which received intravenously 100 mg of RNA-P and RNA-D, respectively. The temperatures were taken again. The control animals showed temperature fluctuations within  $0.1^{\circ}$  to  $0.4^{\circ}\text{C}$ . The tested animals had temperatures fluctuating within the same limits:  $0.1^{\circ}$  to  $0.4^{\circ}\text{C}$ . These results prove the non-pyrogenicity of yeast RNA.

### Example 2. Anti-Inflammatory Action of Nucleic Acids Based on the Model of Thrombocyte Aggregation *in Vitro*

We studied the anti-inflammatory action of nucleic acids on the model of thrombocyte aggregation in vitro by the method of Born (**Born L.V.R. The aggregation of blood platelets by diphosphate and its reversal, Nature, V.94, P.327, 1962**). Venous human blood was taken in silicon tubes of *Becton Dickson*, which contained a 3.8% solution of sodium citrate. In order to receive thrombocytic-rich plasma, citrate blood was centrifuged at 1500 rev/min for 7 minutes. Plasma free of thrombocytes was obtained by centrifuging 2,0 ml of plasma taken from medium layers for 15 minutes at 3000 rev/min. We counted the number of thrombocytes in the thrombocytic-containing plasma, which was later diluted by the thrombocyte-free plasma to the final concentration 200,0-300,0  $\times 10^8/l$ .

An Aggregometer produced by "Tromlite" (Poland) was used for thrombocyte aggregation. In order to induce aggregation, arachidonic acid was diluted in Michaelis



buffer in the proportion 1 mg/ml. Two tubes were inserted in the aggregometer, one of which contained 0.2 ml of thrombocyte-containing plasma, while the other one had 0.2 ml of thrombocyte-free plasma and 0.1 ml of isotonic solution of sodium chloride. After the device was switched on, 0.1 ml of arachidonic acid was added to the tube containing plasma with thrombocytes. Then, the light-transparency of thrombocyte-containing plasma was measured during 5 minutes, which indicated the stage of thrombocyte aggregation.

In a variation of the test for studying the influence of nucleic acids on thrombocyte aggregation, before measuring, the solution of thrombocyte plasma was preliminary incubated for 5 minutes at 37°C with 0.1 ml of the nucleic acid at the corresponding concentration. 0.2 ml of isotonic solution of sodium chloride was added to the tube with thrombocyte-free plasma. After incubation, the device was switched off and 0.1 ml of arachidonic acid was added to the tube with thrombocytic plasma and a nucleic acid. In 5 minutes, measuring was done to determine the final stage of thrombocyte aggregation.

As the aggregation parameter, we used the index of aggregation of cells (IA), which is equal to:

$$I A = \frac{D1 - D2}{D1} \times 100\%$$

D1 – optical density of thrombocyte-containing plasma with the induction of aggregation by the arachidonic acid.

D2 – optical density of thrombocyte-containing plasma, which was preincubated with a nucleic acid and with an induction of aggregation by the arachidonic acid.

Statistical processing of the results was done by Student criteria and with the help of software as described in example 4.1.

The following nucleic acids, were studied: DNA-CT, DNA-EC, tRNA, and total yeast RNA-D in the final concentration  $1 \times 10^{-2}\%$ . Aspirin in the concentration 0.06 mg

per tube, which contained thrombocytic plasma, was also tested as a standard anti-inflammatory agent.

The test results are shown on Table 2 below.

Table 2.

Influence of Nucleic Acids and Aspirin on the Aggregation of Thrombocytes Induced by Arachidonic Acid

	RNA-D	Aspirin	DNA-CT	DNA-EC	t-RNA
M	59,73	38,66	54,45	36,93	52,23
+m	4,24	6,71	3,76	1,88	8,13
		P<0.02	P<0.2	P<0.01	P>0.5

The test results showed that nucleic acids in the concentration  $1 \times 10^{-2}$  % inhibit aggregation of thrombocytes induced by arachidonic acid. Further, Yeast RNA-D in the concentration  $1 \times 10^{-2}$  % inhibited aggregation of the induced thrombocytes almost twice as effectively as aspirin (38.66%): yeast RNA-D showed 59.73% and transport *E.coli* RNA had 52.23%. DNA from chicken erythrocytes acted at the same level as aspirin (36.93%), while DNA from cattle thymus inhibited aggregation of thrombocytes by 54.45%, which is almost at the level of yeast RNA. Since DNA always contain a significant amount of RNA, it is probable that the inhibiting effect of DNA can be attributed to the RNA contained in DNA.

Further, an analysis of the influence of different concentrations of yeast RNA on the aggregation of induced thrombocytes showed that yeast RNA was effective in a wide range of concentrations from 0.1% to  $1 \times 10^{-5}$  % and inhibited aggregation by 78,5% and 14,2%, as shown in Table 3 below.

Table 3.

**Concentration-Dependence of the Influence of Yeast RNA-D on the  
Aggregation of Thrombocytes Induced by Arachidonic Acid**

	RNA 0,1%	RNA $1 \times 10^{-2}$ %	RNA $1 \times 10^{-3}$ %	RNA $1 \times 10^{-4}$ %	RNA $1 \times 10^{-5}$ %
M	78,58	53,08	28,88	43,35	14,23
+m	7,51	3,23	1,63	10,30	4,98
		P<0.01	P<0.001	P<0.01	P>0.001

Still further, it was demonstrated that the inhibiting effect on aggregation depends on the purity of yeast RNA and its sodium salt, as shown on Table 4 below.

Table 4.

**Influence of Yeast RNA-P, -PN and -F on the Aggregation of Thrombocytes  
Induced by Arachidonic Acid**

Conc. 0.1%	RNA-P	RNA-PN	RNA-F
M	84,09	45,96	57,90
+m	3,77	8,96	9,58
		P<0.001	P<0.02
Conc. $1 \times 10^{-2}$ %	RNA-P	RNA-PN	RNA-F
M	71.91	55.44	60.90
+m	8,45	8,04	10,39
		P<0.2	P>0.5
Conc. $1 \times 10^{-3}$ %	RNA-P	RNA-PN	RNA-F
M	29,76	3,72	18,26
+m	5,36	2,40	5,46
		P<0.001	P<0.1

Table 4 shows that RNA-F containing protein admixtures and lower levels of nitrogen and phosphorus content acted less effectively in the range of concentrations from  $1 \times 10^{-1}$  % to  $1 \times 10^{-3}$  %. For example, at its highest concentration, RNA-F inhibited thrombocytic aggregation by 57%, whereas at its lowest concentration, inhibition was only 22.7%. At the same time, well-purified RNA-P inhibited thrombocytic aggregation by a third more effectively, accordingly, by 84% and 29.7%. Also, when RNA was transformed into its sodium salt, the anti-aggregate properties decreased dramatically. Thus, RNA-PN, at its highest concentrations, was only half as effective (44.4%) as the

acid form, while at its lowest concentration, RNA-PN did not show any anti-aggregate properties.

Therefore, based on the model of aggregation of thrombocytes induced by arachidonic acid, it was demonstrated that RNA compounds and, especially, purified yeast RNA, have pronounced anti-aggregate properties in a wide range of concentrations, which indicates their anti-inflammatory action.

### Example 3. Anti-Inflammatory Action of Nucleic Acid Based on the Model of Erythrocyte Membrane Stabilization *in Vitro*

The membrane-stabilizing and anti-radical actions of nucleic acids were evaluated in rat erythrocytes in tests *in vitro*. Erythrocytal membranes were damaged by nitrite anion, a stable metabolite of nitric oxide, which causes oxide injuries in the protein (especially, hemoglobin) and lipid components of the membrane.

In order to evaluate the membrane-stabilizing action of nucleic acids against the influence of free radicals, we calculated the acid resistance of normal rat erythrocytes separated from blood plasma. Rat erythrocytes were rinsed thrice in the cold (4°C) solution of 0,15M of NaCl. The layers of leukocytes and thrombocytes were removed. Acid lysis of the remaining erythrocytes was induced by adding 10 µl of the suspension, which was diluted to the concentration of erythrocytes ( $0,7 \times 10^6$  cells per 1 ml of iso-osmotic medium), and which contained 0,14M of NaCl, 0.01M of the citrate-phosphate buffer pH=2.5, different concentrations (10 or 100 µg) of nucleic acids, and a stable concentration of nitric sodium, 250 µg per 1 ml, to initiate the oxide damage of erythrocytes.

Erythrocytal lysis was initiated by adding 1 ml 0,004N HCl; changes in existence were recorded at 750 nmol. The method of calculation is explained in Example 6.3. It was demonstrated that yeast RNA-D in the doses of 10 and 100 µg increased the level of total resistance of the erythrocytes from 288 units (control value recorded for the influence

of  $\text{NaNO}_2$  without yeast RNA) to 449 units (yeast RNA concentration 10  $\mu\text{g}$ ) and 437 units (yeast RNA concentration 100  $\mu\text{g}$ ), which is close to norm (475 units). RNA-PN increased total resistance to 328 units in the dose of 10  $\mu\text{g}$  and to 415 units in the dose of 100  $\mu\text{g}$ . RNA-P increased total resistance to 315 units in the dose of 10  $\mu\text{g}$  and to 462 units in the dose of 100  $\mu\text{g}$  (maximally close to the normal level of this indicator). RNA-F increased total resistance to 338 units in the dose of 10  $\mu\text{g}$  and, on the contrary, somewhat decreased (to 271 units) in the dose of 100  $\mu\text{g}$ .

DNA-CT increased total resistance to 338 units in the dose of 10  $\mu\text{g}$  and to 654 units in the dose of 50  $\mu\text{g}$  (which is double the control value and even greater than norm (without harmful influence of  $\text{NaNO}_2$ )). In the dose of 100  $\mu\text{g}$ , however, its effect was the opposite – membrane-stabilizing, which was shown by a decreased total resistance to 158 units, which is almost half the control value.

DNA-EC in the dose of 100  $\mu\text{g}$  did not change acid resistance of erythrocytes in our oxide-damage model. In the dose of 10  $\mu\text{g}$ , it increased acid resistance to 408 units, which is a little lower than the calculated protector action of RNA-D (449 units in the dose of 10  $\mu\text{g}$ ).

Therefore, exogenous DNA, regardless of their origin, have significant anti-stabilizing influence on cellular membranes. Since they damage cellular membranes, they cannot be used as drugs or food supplements.

The preparation of t-RNA in both doses (10  $\mu\text{g}$  to 279 units and 100  $\mu\text{g}$  to 296 units  $\mu\text{g}$ ) did not influence the acid resistance of erythrocytes.

The tests show that yeast RNA, when tested in vitro, shows membrane-stabilizing and anti-radical properties which depend on its form, origin, and purity. Well-purified yeast RNA-P, whose anti-inflammatory properties were studied more in detail, showed the best effectiveness.

Example 4. Anti-Inflammatory Action of Nucleic Acid Based on the Model of Local Inflammation Provoked by Carrageenan (LPS)

Example 4.1. Action of Yeast RNA on Swelling in the Model of Local Inflammation Provoked by Carrageenan (LPS) *in Vivo*

To study the anti-inflammatory action of drugs, we used the model of local inflammation in mice. Inflammation in BALB-line mice was modeled with the help of carrageenan, a classical phlogogenic agent. 30 minutes before the injection, the mice were injected intra-abdominally with drug, which was dissolved in 2 mg of physiological solution (PS). Carrageenan (LPS) produced by Serva Fein Biochemica (Germany ) was prepared in the form of a 1% solution in PS. The obtained viscous solution, 40 mcl, was injected subplantally in the left back leg. The right, intact leg was taken as control. 4 hours later after the carrageenan injection, the mice were killed via decapitation, and their back legs were detached from the bodies on the same level, a little higher the ankles. After that, the legs were carefully weighted, with 1 mg accuracy. Obtained results were statistically processed by the MultiFac 2.2. SPSS 8/0 software. The anti-inflammatory effect of the drug was calculated by the formula:

$$\text{Percentage of reduction of inflammation} = \frac{V_k - V_o}{V_k} \times 100\%$$

$V_k$  – average increase of volume (mass) of the swollen leg in control mice

$V_o$  – average increase of volume (mass) of the swollen leg in treated mice

In the first test, mice were divided into 8 groups. The first group consisted of control animals, which were injected intra-abdominally with 2 ml of PS. Also, 40 ml of PS was injected in the left leg. This group was studied to determine the influence of injection on the course of inflammation in a leg. The second group, control with LPS, took 2 mg of PS intra-abdominally and received LPS injections in the left leg. The third

group took 2 mg of aspirin dissolved in PS in the concentration 0.4 mg per mouse. In the fourth, fifth and sixth groups, yeast RNA-D was dissolved in PS in respective concentrations 5, 10, and 15 mg in 2 ml of PS per animal. LPS was injected in the left leg to provoke swelling. The seventh and eighth groups were treated respectively by DNA-TC and DNA-EC, which were injected in the concentration 15 mg per mouse, as explained above for RNA-treated groups.

The right legs were left intact. 4 hours later, the animals were decapitated. Both legs were detached from the bodies and their masses were studied in each group of animals. Results of these tests on the anti-inflammatory action of nucleic acids are presented in Table 5 below.

Table 5.

Influence of Nucleic Acids on Local Inflammation of Mice Legs

Control +PS	Control +LPS	Aspirin	RNA-D 5mg/m	RNA-D 10mg/m	RNA-D 15mg/m	DNA-TC 15mg/m	DNA-EC 15mg/m
0	43.31+ 2.43	35.5+ 2.8	27.4+ 2.05	28.88+ 2.27	20.3+ 3.17	31.8+ 2.59	289
% of inhibition		18.03%	36.74%	47.17%	53.13%	26.58%	33.27%
		P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

As shown in Table 5, aspirin in the administered concentration reduced the development of swelling in mouse legs by 18.03%. This is consistent with the results cited in other papers for this model and proves the adequacy of the modeled inflammation. Aspirin concentrations also correspond to the dose of 20 mg/kg which is currently recommended for a clinical use and which has fewer negative consequences for a long-term use in various forms of inflammatory processes.

Further, the preparation of yeast RNA-D showed a significant anti-inflammatory action, which directly depended on the concentration. In the concentrations 5, 10, and 15 mg per mouse, the drug inhibited swelling by 36.74%, 47.17%, and 53.13% accordingly. The preparation of DNA-TC and DNA-EC also showed some anti-inflammatory action,

though in quite high concentrations (15 mg per mouse), and indicators of the anti-inflammatory action were twice as low (26.58% and 33.27%, respectively).

On the basis of the results, we can conclude that nucleic acids have considerably improved anti-inflammatory properties as compared to aspirin, and yeast RNA has by far the most significant action.

#### Example 4.2. Action of Yeast RNA on Biochemical Indicia in the Model of Local Inflammation Provoked by Carrageenan (LPS) *in Vitro*

The anti-inflammatory action of yeast RNA was compared with the action of aspirin in the dynamics of a developing inflammatory reaction (0<sup>th</sup>, 30<sup>th</sup>, 60<sup>th</sup>, 320<sup>th</sup> min) on mice after LPS injection. We tested the influence of yeast RNA on the activity of NO-synthetase ferment (NOS) in blood plasma and in erythrocytes, as well as on the content in blood plasma of free arachidonic acid and products of its oxide metabolism, carried out in lipoxygenase (leukotriene C4 (LTC<sub>4</sub>)) and cyclooxygenase (thromboxane B<sub>2</sub> (TxB<sub>2</sub>)) ways.

##### Example 4.2.1. Action of Yeast RNA on the Activity of NO-Synthetase

The activity in blood plasma and erythrocytes of the enzyme NO-synthetase was measured by colometric method applied to the outcome of reaction, nitrite anion. (Yan L., Vandivier R.W., Suffredini A.F., Danner R.L., Human polymorphonuclear leukocytes lack detectable nitric oxide synthetase activity. J.Immunol., 15; 153(4): 1825-1834; 1994). The incubation mix (1 ml) consisted of 50 mM of HEPES (pH=7,4), 1,25 mM of CaCl<sub>2</sub>, 1mM of NADPH, 80 mcM FAD, 20 mcM of tetrahydrobiopterine, 13 mcg/ml of calmoduline 1mM of L-arginine, 60 mM of L-valine, 100 units/ml of superoxyddismutase. HEPES is N(2 hydroxyetyl)-1-piperazineethanesulfonic acid by Sigma Chemical Co. (USA), NADPH is beta-nicotinamide adenine di-nucleitide phosphate in reduced form by Sigma Chemical Co. (USA). The reaction was initiated by



adding 0.1 ml of a probe containing 500 microgram of general protein determined by Bredford method. Incubation at 27°C lasted for 60 minutes. The reaction was terminated by adding 0,2 ml of 2N HClO<sub>4</sub>. The mix was centrifuged at 10000g for 10 minutes, and the supernatant liquid was used to determine the content of nitrite-anion (stable metabolite of nitrogen oxide).

Nitrite anion was determined using the reagent of Gris in colometric reaction as decribed in Green et al. (Green L.C., Waagner D.A., Glogowski J. et al., **Analysis of nitrate, nitrite and [15N] nitrate in biological fluids, Anal.Biochem., 126(1): 131-138;1982**). The Gris reagent was prepared by mixing equal parts of 0.1% water solution of naphthylenediaminehydrochloride and 1% solution of sulfanilamide in 5% H<sub>3</sub>PO<sub>4</sub> immediately before the measurement. The measurement was carried out in non-protein aliquots of probe by adding Gris reagent in the 1:1 proportion. In 5 minutes after mixing, the extinction at 543 hm was measured. The quantity of NO<sub>2</sub><sup>-</sup> was measured by standard curve built for NaNO<sub>2</sub>. The test results are presented in Table 6 below.

Table 6.

Action of Yeast RNA and Aspirin on the Activity of NOS in Mouse Blood  
Plasma after Carrageenan Injection  
(in picomol per 1 min per 1 mg of protein; M+-m; n=5)

	LPS (Control)				+Yeast RNA			+Aspirin
	0 min (norm)	30 min	60 min	320 min	30 min	60 min	320 min	320 min
M	18.41	189.45	72.03	110.48	35.40	9.61	107.14	42.24
+m	2.24	21.34	9.25	22.79	7.73	0.96	13.26	4.50
P1		<0.001	<0.001	<0.01	>0.05	<0.01	<0.001	<0.01
P2					<0.01	<0.01	>0.5	>0.05

P1 – certainty of difference with respect to the norm (before LPS injection)

P2 – certainty of difference with respect to the control (without yeast RNA)

Table 6 shows that, without prior injection of yeast RNA control case, a dramatic increase (more than tenfold) of NOS activity in blood plasma was evidenced for 30

minutes after LPS was injected. Then, enzymatic activity decreased with a later minor increase (though at a level much higher than normal).

A prior injection of yeast RNA in mice significantly decreased the rise of NOS activity in blood plasma during the initial stage (30 to 60 minutes) of inflammatory development. This protector property of yeast RNA was not evident on the 320<sup>th</sup> minute of inflammatory development, while an aspirin injection reduced NOS activity exactly during this period of time.

Hence, yeast RNA has a pronounced inhibiting action on activation of the oxide way of L-arginine metabolism after the introduction of LPS, which is expressed by inhibiting the activity of NOS in blood plasma.

Since various isoforms of NOS, both constitutive and inducible, are present in different nucleus cells of blood plasma: neutrophyles, thrombocytes, lymphocytes, and macrophages (Hibbs J.B., Taintor R.R., Vavrin Z., Rachlin E.M., Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* 30; 157(1); 87-94; 1988; Salkowski C.A., Regulation of inducible nitric oxid messenger RNA-expression and nitric oxid production by lipopolysaccharide in vivo: the role of macrophage, endogenous IFN-gamma and TNF receptor-1-mediated signaling. *J.Immunol.* 15; 158(2): 905-912; 1997) we may infer that in the initial stage after LPS introduction (30<sup>th</sup>-60<sup>th</sup> minute), an activation of the constitutive forms (neuronal and endothelial) takes place, while the inducible form (iNOS) of blood macrophages is probably activated in the later period (320<sup>th</sup> minute).

Further, Table 7 below shows the dynamics of changing NOS activity in mice blood erythrocytes after LPS introduction. Control animals showed a minor increase of NOS activity on the 30<sup>th</sup> minute, which was replaced by a significant (almost double) decrease of NOS activity in erythrocytes.

Table 7.

Action of Yeast RNA and Aspirin on the Activity of NOS in Mouse Erythrocytes after Carrageenan Injection (in picomol per 1 min per 1 mg of protein; M<sup>+</sup>-m; n=5)

	LPS (Control)				+Yeast RNA			+Aspirin
	0 min (norm)	30 min	60 min	320 min	30 min	60 min	320 min	320 min
M	4.336	7.768	2.323	2.232	14.245	10.213	1.146	3.613
+m	1.105	0.999	0.383	0.515	1.109	1.924	0.242	0.595
P1		>0.05	>0.1	>0.1	<0.001	<0.05	<0.05	>0.5
P2					<0.01	<0.01	>0.05	<0.2

P1 – certainty of difference with respect to the norm (before carrageenan injection)

P2 – certainty of difference with respect to the control (without yeast RNA)

The same dynamics of modification in NOS activity, or even a more pronounced one, was evidenced in mice erythrocytes after a prior injection of yeast RNA. Thus, an increase of NOS activity (respectively more than three-fold and two-fold) was manifested on the 30<sup>th</sup> and 60<sup>th</sup> minute. A reliable (more than three-fold) decrease of NOS activity in erythrocytes was recorded on the 320<sup>th</sup> minute of LPS action.

Some authors (Chen L.Y., Mehta J.L., Evidence for the presence of L-arginine-nitric oxide pathway in human red blood cells: relevance in the effects of red blood cells on platelet function, J. Cardiovasc. Pharmacol. 32(1): 57-61; 1998) indicate that erythrocytes contain a constitutive, Ca-dependent isoform of NOS. Thus, it is possible that the increased activity of erythrocytal NOS during the initial stage of inflammatory reaction, which was induced by LPS introduction, is caused by increased levels of intercellular calcium in the red cells of blood plasma.

#### Example 4.2.2. Action of Yeast RNA on Oxidizing Metabolism of Arachidonic Acid

The content of free arachidonic acid (AA) was measured by two-dimensional thin-layer chromatography (TLC) as discussed in Tsunamoto et al. (Tsunamoto K.,

**Todo S., Imashuku S. Separation of prostaglandines and thromboxane by two-dimensional thin-layer chromatography. J.Chromatog. 3; 417(2); 414-419; 1987.**

The content of stable metabolite of thromboxane A2 (TXB<sub>2</sub>) was studied in probes by radio-immune method with TXB<sub>2</sub> [<sup>3</sup>H] RIA Kit, by Amersham International PLC (England) (McCann D.S., Tokarsky J., Sorkin R.P., Radioimmuno assay for plasma thromboxane B2. Clin.Chem., 27(8): 1417-1420, 1981). The content of LTC<sub>4</sub> was tested in probes by radio-immune method with LTC<sub>4</sub> [<sup>3</sup>H] RIA Kit by Du Pont Ltd. Hertfordshire, (UK) (Levine L., Morgan R.A., Levis R.A. et al., Radioimmunoassay of the leukotrienes of slow reactivity substance of anaphylaxis. Proc. Natl. Acad. Sci. USA. 78(12): 7692-7696; 1981).

Table 8 below demonstrates the dynamics of changes of free arachidonic acid in mice blood plasma after LPS introduction.

Table 8.

**Action of Yeast RNA and Aspirin on the Content of Free Arachidonic Acid in Mouse Blood Plasma after Carrageenan Injection (in nanomol per 1 mg of protein; M±m; n=5)**

	LPS (Control)				+Yeast RNA			+Aspirin
	0 min (norm)	30 min	60 min	320 min	30 min	60 min	320 min	320 min
M	2.54	2.36	3.34	3.37	1.97	1.60	2.66	2.64
±m	0.26	0.23	0.37	0.11	0.13	0.16	0.20	0.13
P1		<0.1	>0.1	<0.05	>0.05	>0.02	>0.1	>0.1
P2					<0.01	<0.01	<0.02	<0.01

P1 – certainty of difference with respect to the norm (before carrageenan injection)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 8, the control animals demonstrated increased levels of arachidonic acid only on the 320<sup>th</sup> minute after LPS introduction. Yeast RNA evidently decreased AA content in blood plasma on the 60<sup>th</sup> minute of LPS action. A decrease on the 30<sup>th</sup> minute was not evident. On the 320<sup>th</sup> minute after LPS introduction yeast RNA

evidently decreased the content of AA in blood plasma in comparison with the control group.

It is known that free arachidonic acid is produced when membrane phospholipids are hydrolyzed with AA phospholipase, which is activated at increased levels of free ionized calcium (Leslie C.C., Channon J.Y., Anionic phospholipids stimulate an arachinoil-hydrolyzing phospholipase A2 from macrophage and reduce the calcium requirement for activity. *Biochim. Biophys. Acta.* 6; 1045(3), 261-270; 1990) Besides, there are other possible ways of releasing free AA, for example, hydrolysis of cholesterol ethers by cholesteroesterase (Moscat J., Moreno F. Herrero C.,et al., Arachidonic acid releasing systems in pig aorta endothelial cells, *Biochem.Biophys Res.Comm.* 30; 139(3): 1098-1103; 1986). Since, the first way of synthesis of free arachidonic acid is more frequent in inflammatory processes, the test results indicate that yeast RNA possibly inhibits the activity of phospholipase in blood plasma.

Further, Table 9 below shows the action of yeast RNA on the contents of thromboxane B<sub>2</sub>, a stable metabolite of A<sub>2</sub> thromboxane which is produced during oxidizing cyclooxygenase metabolism of arachidonic acid.

Table 9.

Action of Yeast RNA and Aspirin on the Content of Thromboxane in Mouse Blood Plasma after Carrageenan Injection (in picomol per 1 mg of protein; M+-m; n=5)

LPS (Control)				+Yeast RNA				+Aspirin
	0 min (norm)	30 min	60 min	320 min	30 min	60 min	320 min	320 min
M	142.610	415.250	578.775	358.240	394.940	560.813	217.602	153.903
+m	34.210	66.600	123.800	11.150	23.550	67.280	32.270	15.880
P1		<0.1	<0.2	<0.001	<0.001	<0.001	<0.2	>0.5
P2					>0.5	>0.5	<0.001	<0.001

P1 – certainty of difference with respect to the norm (before carrageenan injection)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 9, after LPS introduction, a dramatic increase of TXB<sub>2</sub> pools in mice plasma was evidenced on the 30<sup>th</sup> and, especially, on the 60<sup>th</sup> minute. On the 320<sup>th</sup> minute, the levels of TXB<sub>2</sub> started to drop. Yeast RNA, like aspirin, which is a known inhibitor of the cyclooxygenase metabolism of arachidonic acid (cyclooxygenase and thromboxanesynthetase), intensifies such a decrease of TXB<sub>2</sub> levels after their rapid increase in the early stage of inflammatory processes.

Next, Table 10 shows the dynamics of changes in the contents of peptidoleukotriene C<sub>4</sub>, a metabolite of lipoxygenase oxidation of AA, in mice blood plasma after LPS injection.

Table 10.

Action of Yeast RNA and Aspirin on the Content of Leukotriene C<sub>4</sub> in Mouse Blood Plasma after Carrageenan Injection (in picomol per 1 mg of protein M+-m; n=5)

M +-m	LPS (Control)				+Yeast RNA			+Aspirin
	0 min (norm)	30 min	60 min	320 min	30 min	60 min	320 min	320 min
P1	71.60 10.72	156.64 10.03	266.33 41.09	226.78 7.48	92.18 15.66	227.00 36.12	129.35 19.25	93.99 1.99
P2		<0.001	<0.01	<0.001	<0.5	<0.01	<0.5	<0.1
					<0.02	<0.5	<0.01	<0.001

P1 – certainty of difference with respect to the norm (before carrageenan injection)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 10, the control group of animals showed an increase of LTC<sub>4</sub> contents in the interval between 30<sup>th</sup> and 60<sup>th</sup> minute, with a slight decrease against the normal level on the 320<sup>th</sup> minute. Animals taking yeast RNA showed LTC<sub>4</sub> levels, which were lower than control on the 30<sup>th</sup> and 320<sup>th</sup> minutes of LPS action. Aspirin showed a similar inhibiting action, which was more pronounced than the action of yeast RNA on the 320<sup>th</sup> minute.

In conclusion, the test results above indicate that yeast RNA, not only inhibits the generation of free arachidonic acid after LPS introduction, but also inhibits its oxidation, both through lipoxygenase and cyclooxygenase.

#### Example 5. Anti-Inflammatory Action of Yeast RNA Based on the Model of Ischemia-Reperfusion in Rats

##### Example 5.1. Cardioprotective Action of Yeast RNA

13 white rats with body mass 200-250 g were anesthetized with urethane and received intra-abdominal injections at 1.25g/kg (**Kogan A.H., Modeling the myocardial infarction, M., 1979**). A tracheostome with inserted intubation pipe was placed on the rats. Artificial ventilation of lungs was provided by Vita-1 device. Skin and other tissues down to the intercostal muscles were incised with a 2-3 mm indentation from the middle sternal line. The 4-4.5 cm. incision stretched from the jugular undercut to the sword-shaped appendix. The lower parts of the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> ribs, as well as the intercostal muscle between the 3<sup>rd</sup> and the 4<sup>th</sup> ribs, were dissected by eye scissors. The initial section of the left coronary artery is usually located in the space between left auricle's eye and pulmonary cone.

A strip of myocardium sized 1.5-2 mm x 1-1.5 mm was stitched up with a 3/0 atraumatic needle, while going along the initial section of the artery, which could easily be seen. The revealed ligature was bandaged around the artery and surrounding muscles. Then we started the observation of the initial macro-signs of ischemia and developing infarction. During the first 10-20 seconds of ischemia, the tissue turned pale, especially in the upper portion of heart, and later changed partially or totally to blue (cyanosis). Contractions of the occlusion zone weakened, and it dilated. ECG's at the same standard distance from the extremities had been continuously recorded during the 30 minutes of

ischemia and 60 minutes of reperfusion. 200 mg/kg of yeast RNA and 20 mg/kg of aspirin were injected 30 minutes before the start of ischemia.

To determine the area and size of the post-infarction scar in rats, sections of myocardium were dyed in accordance with the p-nitrobluetetrazolium method (**Mueller B., Maass B., Krause W., Witt W., Limitation of myocardial unperfused area and necrotic zone 24 hours and 7 days after coronary artery ligation in rats by the stable prostacyclin analogue iloprost, Prostaglandins Leucot. Med. 21(3): 331-340; 1986).** After reperfusion, the animals were heparinized (150 IU/kg i.v.) the hearts removed in deep ether anaesthesia and retrogradely perfused with a solution of 0,05 % p-nitroblutetrazolium in phosphate buffer (30 min;100 mmHg;37 C). After 24 hours fixation in formaldehyde solution the ventricles were weighed, transversely sectioned into 5 slices each, and an unstained area was divided from the stained myocardium and weighed. The necrotic zone was calculated.

Analysis of the necrosis zone 60 minutes after ischemia determined that the risk zone in the left ventricle of the heart constituted  $33.3 \pm 3.4\%$  of the left ventricle mass. In the control group, the infarction zone constituted  $60.3 \pm 3.8\%$  of the risk zone. Yeast RNA injection 30 minutes before the start of infarction on 41% decreased the proportion between infarction and risk zones to 32.1%.

Analysis of ECG in ischemia-reperfusion of myocardium in rats showed that a prior injection of yeast RNA compound decreased the amount of extrasystols. In only one of the five rats in this group, 4 extrasystols were detected. In the control group, which consisted of rats not treated by yeast RNA, we registered extrasystols in 3 rats, on average  $8.7 \pm 1.7$ . The intervals of paroxysmal tachycardia in the control group lasted longer: 2 out of five rates had the episodes lasting for  $4.2 \pm 1.3$  sec on average.

In the group treated by yeast RNA only one rat out of five had an interval of paroxysmal tachycardia, which lasted for 1.5 sec. ECG analysis showed that yeast RNA improves the heart function in ischemia-reperfusion of myocardium, stabilizes the



leading heart system, and has a significant anti-arrhythmic action by decreasing the quantity of extrasystols and shortening the paroxysmal tachycardia interval.

In conclusion, these test results show that yeast RNA has a pronounced cardio-protector action in infarction of myocardium in rats.

Example 5.2. Action of Yeast RNA on the Activity of Myeloperoxidase in the Ischemized Part of Myocardium

The mieloperoxidase activity (MPA) was studied in myocardium using the method of Bradley et al. (Bradley P.P., Priebet D.A., Christensen R.D. et al., **Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker**, J. Invest. Dermatol., 78(3): 206-209; 1982) in the modification by Griswold et al. (Griswold D.E., Hillegass L.M., Hill D.E. et al., **Method for quantification of myocardial infarction and inflammatory cell infiltration in rat cardiac tissue**, J. Pharmacol. Methods, 20(3): 225-235, 1988). For this purpose, the heart was extracted and rinsed in physiological solution, which was cooled to 0°C. After rinsing, a section of myocardium (1 g of the tissue) in the central zone of ischemia was cut out and frozen to -30°C. The final fraction was prepared as a 10% haemogenate with extractive buffer containing 0.5% hexadecyltrimethyl ammonium bromide (pH 6,0) at room temperature. Afterwards, it was centrifuged for 20 minutes at 4°C and 12000 g.

The upper fraction (30 microliters) was used for a reaction with 0.167 mg/ml of O-dianisidine in 50 millimole/l of potassium phosphate buffer (pH 6,0). The reaction was launched with adding 0.005% solution of H<sub>2</sub>O<sub>2</sub>. The reaction had been continuously tested for 5 minutes at 460 nm wave length, and with readings taken every minute. A chart indicating the readings was prepared. A unit of MPA was defined as the quantity of ferment, which destroys 1 micromole/min H<sub>2</sub>O<sub>2</sub> at 25°C. The data was calculated as MPA per 1 gram of tissue.



### Example 5.3. Action of Yeast RNA on the Activity of NOS in Ischemia Cases

The tests were conducted on rats with an infarct of myocardium experimentally induced by occlusion of the coronary artery for 30 minutes. Blood was taken from the coronary artery and from the heart, which was divided into the intact zone, border zone, and infarction zone. The activity of NOS ferment was measured in different heart zones and in blood. Also, we measured the contents of free arachidonic acid (heart and blood) and products of its oxidizing metabolism (blood). The test results are shown in Table 11 below.

Table 11.

Action of Yeast RNA on the Activity of NOS in Rat Heart in Ischemia  
(in picomol per 1 mg of protein; M<sup>+</sup>-m; n=5)

	Ischemia 30 min (Control)				Ischemia 30 min+Yeast RNA		
	Intact (norm)	Border zone	Infarction zone	Together	Border zone	Infarction zone	Together
M	46.500	259.31	185.626	129.65	59.634	115.122	122.63
+m	7.000	0	48.635	5	11.649	40.509	0
		60.683		30.341			26.413
P1		<0.01	<0.05	<0.2	<0.5	<0.1	>0.05
P2					<0.2	<0.5	<0.05

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

The data in Table 11 demonstrates that, during a short-term ischemia, the activity of NOS increased more than three-fold in the infarction zone ( $115 \pm 40$  and  $186 \pm 49$  pmol/min on 1mg of protein accordingly, in the test and control groups). Hence, yeast RNA almost completely normalized the activity of NOS in the border zone of ischemic heart infarction, which may be one of the mechanisms of its cardio-protecting action.

Since cardiomyocytes contain both the inducible NOS isoform and its constituent isoforms (Balligand J.L., Kobzik L., Han X., et al., Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III)

nitric oxid synthetase in cardiac myocytes, *J. Biol.Chem.*, 16; 270(24); 14582-14586; 1995; Peng H.B., Spiecker M., Liao J.K. Inducible nitric oxid: an autoregulatory feedback inhibitor of vascular inflammation, *J.Immunol.* 15; 161(4): 1970-1976; 1998; Oddis C.V., Simmons R.L., Haffler B.G., Finkel M.S. cAMP enhances inducible nitric oxid synthase mRNA stability in cardiac myocytes, *Am. J. Physiol.* 269(6): H2044-2050; 1995), taking into account the short period (30 minutes) of ischemia, it is inferred that yeast RNA inhibits the constituent isoform of NOS. At the same time, since iNOS is present, yeast RNA may also act as an inhibitor of activity of inducible NOS in ischemic cardiomyocytes.

Further, the influence of yeast RNA on NOS activity in rat blood in ischemia is shown in Table 12 below in Example 5.4. The test results in Table 12 show that, unlike in the heart, the activity of NOS in the blood of control animals in ischemia decreased twice (14,22+1,43 and 30,35+3,40 pmol per 1 mg of protein accordingly in ischemia and normoxia), which is usual for hypoxia (Arnet W.A., McMillan A., Dinerman J.L. et al., Regulation of endothelial nitric oxid synthase during hypoxia, *J.Biol.Chem.* 271(25): 15069-15073; 1996). Introduction of yeast RNA almost completely normalized NOS activity in rat blood after a 30-minute ischemia.

**Example 5.4. Action of Yeast RNA on the Oxidizing Metabolism of Arachidonic Acid in Ischemia Cases**

Table 12 below shows the content of NOS and free arachidonic acid in the blood of normal and ischemic animals.

Table 12.

**Action of Yeast RNA on the Activity of NOS and Content of Arachidonic Acid in Rat Blood in Ischemia**

	NOS Activity in picomol per 1 mg protein; M+-m; n=5			Content of free Arachidonic acid (nmol/1mg of protein)		
	Norm	Ischemia 30 min	Ischemia +Yeast RNA	Norm	Ischemia 30 min (control)	Ischemia +Yeast RNA
M	30.35	14.22	26.45	0.77	0.24	0.48
+m	3.40	1.43	3.73	0.13	0.04	0.02
P1		<0.01	<0.5		<0.01	>0.05
P2			<0.05			<0.01

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 12, the control group of animals demonstrated decreased levels of free AA more than three-fold ( $0.77 \pm 1.43$  and  $30.35 \pm 3.40$  nmol/min on 1 mg of protein accordingly in normoxia and ischemia cases). The introduction of yeast RNA somewhat normalized the content of AA, by increasing it twice against the control value ( $P < 0.001$ ).

Table 13 indicates the content of free arachidonic acid in different heart zones in ischemia cases.

Table 13.

**Action of Yeast RNA on the Content of Free Arachidonic Acid in Rat Heart in Ischemia  
(in nmol per 1 mg of protein; M-m; n=5)**

	Ischemia 30 min (Control)				Ischemia 30 min+Yeast RNA		
	Intact (norm)	Border zone	Infarction zone	Together	Border zone	Infarction zone	Together
M	4.827	9.910	9.716	9.813	7.270	8.530	7.900
+m	0.378	1.003	0.947	0.919	0.456	0.741	0.493
P1		<0.01	<0.01	<0.001	<0.01	<0.01	<0.01
P2					>0.05	>0.05	>0.5

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 13, the control group of animals demonstrated a reliable (more than two-fold,  $P<0.01$ ) increase of AA levels both in the border and infarction zones of rat hearts. The introduction of yeast RNA somewhat decreased the level of arachidonic acid in both heart zones, but the difference was not evident ( $P>0.05$ ).

Table 14 below shows the action of yeast RNA on the levels of eicosanoids in rat blood in ischemia cases.

Table 14.

Action of Yeast RNA Compound on Eukosanoids in Rat Blood in Ischemia  
(in picomol per 1 mg of protein; M-m; n=5)

	Thromboxane B2			Leucotriens C4		
	Norm	Ischemia 30 min (control)	Ischemia +Yeast RNA	Norm	Ischemia 30 min (control)	Ischemia +Yeast RNA
M	53.00	130.72	62.49	24.10	39.62	25.69
+m	10.67	33.92	7.98	3.94	10.18	4.04
P1		<0.05	>0.5		<0.2	>0.5
P2			<0.1			<0.5

P1 – certainty of difference with respect to the norm (before ischemia)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 14, the control group of animals demonstrated more increased levels of products of cyclooxygenase reaction of  $TBX_2$  (more than two-fold, but the difference is not evident –  $P>0.05$ ) rather than the levels of the product of lipxygenase reaction  $LTC_4$  ( $P<0.2$ ). The introduction of yeast RNA almost completely normalized that content of eicosanoids in rat blood in ischemia cases.

In conclusion, in addition to the modulating influence on NOS activity in ischemia (inhibition in cardiomyocytes and, on the contrary, increase in blood), the cardio-protecting action of yeast RNA may also be mediated by modulating the oxidizing metabolism of arachidonic acid.

Example 6. Anti-Inflammatory Action of Yeast RNA Based on the Model of Auto-Immune Pathology Model (Adjuvant Arthritis) *in Vivo*

Adjuvant arthritis develops after rats are injected with Freud's adjuvant and is a part of the generalized process, which is accompanied by the impairment of bone and connecting tissues. Morphological tests show, that during the development of adjuvant arthritis, inflammatory-degenerative changes emerge in tissues surrounding the joint as well as inside the articular bursa and in joint cartilages. It is believed that this inflammatory reaction has all the properties of an immunologic process and constitutes a delayed immune reaction to a microbe antigen. The pathological process of adjuvant arthritis is very similar to arthritis in humans.

Example 6.1. Action of Yeast RNA in an Auto-Immune Pathology Model (Adjuvant Arthritis)

Adjuvant arthritis was modeled in rat males according to Courtright et al. (Courtright L.J., Kuzell W.C., Sparing effect of neurological deficit and trauma on the course of adjuvant arthritis in the rat, *Ann. Reum. Dis.* 24(4): 360-368; 1965). Control animals received a single hypodermic dose of 0.1 ml of standard Freud's adjuvant in the distal part of the tail. Adjuvant arthritis developed on the 14-20<sup>th</sup> day after the injection. Arthritis symptoms were determined by X-rays: a darkened area and shadows around the joints of back legs imply a starting impairment of the joint and gristle tissue.

In the test group yeast RNA was diluted in a 0.9% concentration of NaCl, was injected intra-abdominally in the concentration 100 mg per rat, a day before the injection of Freud's adjuvant. Yeast RNA has been also introduced after adjuvant's injection in three series within 4 days with three-day intervals.

Results of the analysis showed that arthritis in the control group started to develop on the 14<sup>th</sup> day and was manifested by exudative-proliferate growth of the synovial capsule and gristle impairment. On the 20<sup>th</sup> day, a hardening of tissue around the joint was witnessed and fibrosis of the synovial capsule started. On the 30<sup>th</sup> day, the ruining of gristle becomes evident. In the test group, which took yeast RNA, no signs of arthritis were witnessed for 20 days. Arthritis symptoms, similar to the ones witnessed in the control group on the 14<sup>th</sup> day, appeared only on the 30<sup>th</sup> day.

During the development of adjuvant arthritis, back legs became larger in the control animals. In particular, on the 30<sup>th</sup> day in the control group, the size of back legs evidently increased by 1.04 millimeter ( $4.9 \pm 0.13$  in comparison with  $3.86 \pm 0.1$  at the beginning of experiment). In the test group, legs grew only by 0,24 millimeter ( $4,1 \pm 0.11$  on the 30<sup>th</sup> day of experiment from  $3.96 \pm 0.08$  at the beginning of experiment). Hence, yeast RNA delays the development of adjuvant arthritis, which is also supported by a decreased growth rate of back legs.

Example 6.2. Action of Yeast RNA on the Activity of NOS in Rat Blood in an Auto-Immune Pathology (Adjuvant Arthritis)

The activity of NOS was evaluated in the blood of normal rats and, on the 3<sup>rd</sup>(I), 8<sup>th</sup>(II), and 14<sup>th</sup>(III) day in the course of an auto-immune pathology in the control group of rats (i.e., not taking yeast RNA) and in the test animals, which were injected with yeast RNA. The results are shown in Table 15 below.



Table 15.

Action of Yeast RNA Compound on the Activity of NOS in Rat Blood in the Dynamics of Adjuvant Arthritis (in picomol per 1 min per 1 mg of protein;  $M \pm m$ ;  $n=5$ )

	Norm	Control			+Yeast RNA		
		I 3 <sup>rd</sup>	II 8 <sup>rd</sup>	III 14 <sup>rd</sup>	I 3 <sup>rd</sup>	II 8 <sup>rd</sup>	III 14 <sup>rd</sup>
M	30.650	236.760	24.340	111.540	70.000	40.660	33.960
+m	7.352	76.418	8.596	15.777	9.245	5.052	6.036
P1		<0.05	>0.5	<0.01	<0.02	<0.5	>0.5
P2					<0.02	<0.1	<0.01

P1 – certainty of difference with respect to the norm (in adjuvant arthritis)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 15, the control group of animals showed a substantial increase of NOS activity on the 3<sup>rd</sup> and 14<sup>th</sup> day of auto-immune pathology in comparison with norm ( $30,65 \pm 7,35$  picomol per 1 min per 1 mg of protein in norm,  $236,76 \pm 76,42$  picomol per 1 min per 1 mg of protein on the 3<sup>rd</sup> day, and  $111,54 \pm 15,78$  picomol per 1 min per 1 mg of protein on the 14<sup>th</sup> day). Such a significant increase in the activity of NOS indicates that activity of the inducible NOS-isoform (iNOS), whose synthesis is initiated by anti-inflammatory cytokines  $\text{INF-}\gamma$ ,  $\text{IL-1}\beta$ ,  $\text{TNF-}\alpha$ , et. al., is the main compound in the calculated activity of NOS.

In the period between the 3<sup>rd</sup> (initiation of the auto-immune process) and 14<sup>th</sup> day (development of pathology), we observed a normalization in the activity of NOS in blood ( $24,34 \pm 8,60$  pmol per 1 min per 1 mg of protein). This may probably be attributed to the activated protective reaction of body, and could be induced by inhibition of the expression of NOS as well as by modulation of the stability of its mRNA, or by inhibiting the process of its translation.

In the group of animals which took yeast RNA, initiation of the auto-immune process (on the 3<sup>rd</sup> day) was accompanied by a much smaller (in comparison with the control group) increase in the activity of NOS in blood ( $70,00 \pm 9,24$  pmol per 1 min per 1

mg of protein against  $236,76 \pm 76,42$  pmol per 1 min per 1 mg of protein). Moreover, the activity of NOS decreased progressively over the next period in development of auto-immune process ( $40,66 \pm 5,05$  pmol per 1 min per 1 mg of protein on the 8<sup>th</sup> day and  $33,96 \pm 6,04$  pmol per 1 min per 1 mg of protein on the 14<sup>th</sup> day).

Therefore, our tests on changes in the activity of NOS in rat blood in the course of an auto-immune process lead to the conclusion that yeast RNA is effective in decreasing the activity of iNOS in the course of an auto-immune process, both during its initiation and in the chronic stage. This property allows the use of yeast RNA in pathological conditions which are accompanied by iNOS induction: inflammatory processes, diabetes, atherosclerosis, tumour, hepatitis, infections, neuro-degenerate diseases (Parkinson's disease, Alzheimer's disease, multiple sclerosis, encephalitis), and others.

### Example 6.3. Membrane-Protecting Action of Yeast RNA

The tests were conducted *in vivo* on the model of a chronic auto-immune process, which was accompanied by generation of a great quantity of free radicals (especially, nitric oxide) during the early stage of initiation. The membrane-protecting action of yeast RNA was studied by evaluating acid resistance of erythrocytes in the course of an auto-immune process. Acid resistance characterizes the wholeness of erythrocytal membranes. It increases in the chronic stage of different pathologies and decreases in the acute stage of development (process of initiation). For example, in the early period of development in inflammations, free-radical processes, which are induced by a generation of free radicals of oxygen and nitrogen, including nitric oxide generated by the inducible isoform of NOS (iNOS), are highly activated.

The level of damage in erythrocytes under the influence of various harmful factors in the course of an auto-immune process was evaluated by kinetic indicators of hemolysis, induced by a pH decrease in the environment. Kinetic indicators of hemolysis were recorded; the number of damaged cells was determined spectrophotometrically in

equal periods of time (30 s) by changes in the value of integral light dispersion of erythrocytal suspension ( $\lambda=750$  nmol). Absorption spectra were registered by a spectrometer SF-26 (Russia). Acid lysis of erythrocytes was initiated by adding 10  $\mu$ l of blood, which was diluted 20 times in the isotonic medium 0,14 mol of NaCl + 0.01 mol of the citrate-phosphate buffer with pH=2,0-3,5 (volume: 1 ml; density of erythrocytes in suspension:  $0,7 \times 10^6$  cells per ml). For such densities, the value of integral light dispersion of erythrocytes depends on the count, size, and shape of cells and is proportional to the number of cells in suspension.

Results are represented in the diagram of acid hemolysis of erythrocytes in Table 16 below, as the integral parameter of this process: total number of acid resistance of erythrocytes was calculated by summing up the products of the number of cells  $a_i$  which hemolyzed over the period of time  $a_i$  and  $t_i$  (total resistance (I) =  $\sum a_i \bullet t_i$ ).

Decreased extinction levels on hemolysis diagrams represent the succession of erythrocytes with increased resistances entering hemolysis. Extinction starts decreasing usually 1.5 – 2 min later after a hemolytic injection (1 ml 0.004N HCl, which was prepared from 0.1N HCl and checked by titration). A lag-period of hemolysis is caused by a pre-hemolysis change in the form of erythrocytes (spherulation). Hemolysis of a single erythrocyte does not exceed 10 seconds. Hence, a 30-second interval between the measurements of existence levels excludes the possibility of counting twice the same erythrocyte undergoing lysis. It follows that, by the photometric registration of hemolysis kinetics, we can calculate, from the derived series of existences with intervals 30 seconds, the percentage of distribution of erythrocytes by resistance groups.

The change of existence from the beginning of hemolysis ( $E_b, t_b$ ) to its final completion ( $E_e, t_e$ ) is proportional to the number of all cells involved in hemolysis (100%), hence:

$$\Delta E = E_e - E_b = 100\%.$$

This total quantity of erythrocytes which undergo hemolysis (100%) consists of the quantity of erythrocytes which undergo hemolysis each 30 seconds ( $E_{i+1}-E_i$ ) in the interval  $t_e-t_b$  = duration of hemolysis:

$$\Delta E = \Sigma E_{i+1} - E_i = 100\%.$$

The results are shown in Table 16 below.

Table 16.

Action of Yeast RNA Compound on the Acid Resistance of Erythrocytes in the Dynamics of Adjuvant Arthritis (Total Resistance; M+-m n=5)

	Norm	Control			+Yeast RNA		
		I 3 <sup>rd</sup>	II 8 <sup>rd</sup>	III 14 <sup>rd</sup>	I 3 <sup>rd</sup>	II 8 <sup>rd</sup>	III 14 <sup>rd</sup>
M	712.333	95.400	448.600	1013.800	372.600	638.800	565.800
+m	85.429	37.776	95.843	290.509	72.667	78.903	80.244
P1		<0.01	<0.1	<0.5	<0.05	>0.5	>0.5
P2					<0.02	<0.2	<0.2

P1 – certainty of difference with respect to the norm (in adjuvant arthritis)

P2 – certainty of difference with respect to the control (without yeast RNA)

As shown in Table 16, there is total resistance of non-showered rat erythrocytes in the course of an auto-immune process. This indicator is equal to 712±85 for normal erythrocytes. During the initiation of an auto-immune process, total resistance of erythrocytes decreased 7 times and constituted 95±38 units. It was gradually increasing in the course of pathology and reached 1114±290 units on the 14<sup>th</sup> (III) day.

Such a significant decrease in the acid resistance of erythrocytes indicates substantial changes in plasmatic cellular membranes, which is perhaps due to the oxidation of protein and lipid membrane components by free radicals, including nitric oxide, which are actively generated in this period, and in plasma, from which we can infer a modulation in the contents of free cholesterol, polyamines, and other stabilizers, as well as increased levels of destabilizers, such as polyunsaturated free fatty acids.

Animals which took yeast RNA during the initiation of an auto-immune process did not have such decreased acid resistance of erythrocytes as in the control group. Total resistance was equal to  $373 \pm 73$  units, which, though lower than the norm ( $P < 0,05$ ), is greater than in the control group ( $P < 0,05$ ). During the later periods in development of auto-immune pathology, total resistance of erythrocytes in animals taking yeast RNA was at the normal level.

Therefore, yeast RNA has immune-stabilizing action. Taking into account the main mechanisms of damage in this pathology, which are oxide stress and damage of plasmatic membrane components by free-radicals, we can also conclude that yeast RNA is anti-radical.

## CLAIMS

1. A method for the treatment of inflammation or inflammatory-related disorder comprising administering to a mammal in need of such treatment an amount effective to ameliorate the symptoms of inflammation or inflammatory-related disorder of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

2. A method of stabilizing damaged cellular membranes which comprises administering to a mammal having damaged cellular membranes an amount effective to stabilize said damaged cellular membranes of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

3. A method of inhibiting oxidation of components of cell membranes of a mammal, which comprises administering to a mammal in need of such treatment an amount effective to inhibit oxidation of components of cell membranes of the mammal of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

4. A method of normalization of NO-synthetase activity in a mammal, which comprises administering to a mammal in need of such treatment an amount effective to normalize NO-synthetase activity in the mammal of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

5. A method of inhibiting thrombocyte aggregation, which comprises administering to a mammal in need of such treatment an amount effective to inhibit thrombocyte aggregation of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.



14. A method in accordance with claim 1, wherein the inflammatory-related disorder is stroke.

15. A method in accordance with claim 1, wherein the inflammatory-related disorder is arthritis.

16. A method in accordance with claim 1, wherein the inflammatory-related disorder is allergy.

17. A method in accordance with claim 1, wherein the inflammatory-related disorder is pain.

18. A method in accordance with claim 1, wherein the inflammatory-related disorder is fever.

19. A method in accordance with claim 1, wherein the inflammatory-related disorder is swelling.

20. A pharmaceutical composition for the treatment or the prevention of inflammation or inflammatory-related disorder, comprising ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent.

21. A pharmaceutical composition in accordance with claim 20, wherein said ribonucleic acid has a nitrogen content is more than 14.5% by weight.

22. A pharmaceutical composition in accordance with claim 20, wherein said ribonucleic acid has a phosphorus content of more than 8.5% by weight.



## ABSTRACT

The present invention concerns a compound consisting of RNA, in particular RNA extracted from yeast, a pharmaceutical composition comprising such RNA and a method for the treatment of inflammatory and inflammatory-related disorders comprising administering to a patient in need of such treatment a pharmaceutical composition comprising an amount effective to ameliorate the symptoms of inflammation or inflammatory-related disorder of ribonucleic acid and a pharmaceutically acceptable vehicle, carrier, or diluent. The exogenous yeast RNA used in the present invention has a pronounced membrane-stabilizing action in a wide range of concentrations. At the same time, yeast RNA normalizes metabolism of arachidonic acid and levels of its key metabolites, thromboxane and leukotriene. Its anti-inflammatory action is accompanied by normalization of the activity of NO-synthetase and anti-oxidant activity.

## Declaration for U.S. Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled  
(Insert Title) COMPOUND, COMPOSITION AND METHOD FOR THE TREATMENT OF INFLAMMATORY AND INFLAMMATORY-RELATED DISORDERS  
the specification of which is attached hereto unless the following is checked

☐ was filed on \_\_\_\_\_ as United States Application Number or PCT International Application Number \_\_\_\_\_  
and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 (a) - (d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application for which priority is claimed:

(List prior  
foreign  
applications.  
See note A on  
back of this  
page)

	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed ___ Yes ___ No
	(Number)	(Country)	(Day/Month/Year Filed)	___ Yes ___ No
	(Number)	(Country)	(Day/Month/Year Filed)	___ Yes ___ No
	(Number)	(Country)	(Day/Month/Year Filed)	___ Yes ___ No

(See note B on back of this page)

\_\_\_ See attached list for additional prior foreign applications

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(List Prior U.S.  
Applications)

(Appln. Serial No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)
(Appln. Serial No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)
(Appln. Serial No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

James E. Armstrong, III, Reg. No. 18,366; William F. Westerman, Reg. No. 29,988; Ken-Ichi Hattori, Reg. No. 32,861; Le-Nhung McLeland, Reg. No. 31,541; Ronald F. Naughton, Reg. No. 24,616; John R. Pegan, Reg. No. 18,069; William G. Kratz, Jr., Reg. No. 22,631; James P. Welch, Reg. No. 17,379; Albert Tockman, Reg. No. 19,722; Mel R. Quintos, Reg. No. 31,898; Donald W. Hanson, Reg. No. 27,133; Stephen G. Adrian, Reg. No. 32,878; William L. Brooks, Reg. No. 34,129; John F. Carney, Reg. No. 20,276; Edward F. Welsh, Reg. No. 22,455; John P. Kong, Reg. No. 40,054; Luke A. Kilyk, Reg. No. 33,251; and Nicolas E. Seckel, Reg. No. 44,373.

Please direct all communications to the following address: ARMSTRONG, WESTERMAN, HATTORI,  
McLELAND & NAUGHTON  
1725 K Street, N.W., Suite 1000  
Washington, D.C. 20006  
Telephone: (202) 659-2930 Fax: (202) 887-0357

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See note C  
above)

Full name of sole or first inventor (given name, family name) Zenoviy TKACHUK

Inventor's signature *B. Tkachuk* Date 03.23.2000

Residence 1250 I Street NW Suite 1105 Citizenship Ukrainian

Post Office Address Washington, D.C. 20005

Full name of third inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of third inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of fourth inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of fifth inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of sixth inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of seventh inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_

Full name of eighth inventor (given name, family name) \_\_\_\_\_

Inventor's signature \_\_\_\_\_ Date \_\_\_\_\_

Residence \_\_\_\_\_ Citizenship \_\_\_\_\_

Post Office Address \_\_\_\_\_